Physical and Linkage Mapping of Genetic Markers and Genes Associated with Sex Determination in Tilapia (*Oreochromis spp*.)

A thesis submitted for the Degree of Doctor in Philosophy

By

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DEDICATED
To

JULIETA and ADLER

My beloved wife and son

"The future belongs to those who believe

in the beauty of their dreams"

-- ELEANOR ROOSEVELT –

DECLARATION

I herby declare that this thesis has been composed entirely by me based on my own investigation. It has neither been accepted nor submitted for any other degrees. All the information from other sources has been properly acknowledged.

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DATE _____

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ABSTRACT

In order to combine previous observations from different sources on sex determination, and to identify sex chromosomes including the major sex determination locus in Nile tilapia, physical and genetic maps based on sex-linked markers and genes (such as sex-linked AFLPs, microsatellites, ovarian aromatase and DMO genes) were integrated and anchored.

An accurate physical map using FISH techniques on mitotic cells was developed based on a previous map and 23 tilapia BAC clones previously assigned to linkage groups (LGs) 1, 3, 6, 7, 10 and 12; and on meiotic cells, 2 BAC clones containing the SLAM *OniY*227 and the dmrt4 gene were mapped. The six linkage groups were then assigned to different chromosomes, but surprisingly, the putative sex LG1 was located to a small submetacentric chromosome and not to the larger subtelocentric chromosome 1, where LG3 was assigned instead. The other LGs were assigned to different chromosomes and oriented with respect to the centromeres. A detailed comparison of the physical distribution of markers on chromosome 1 with respect to LG3 revealed a suppression in recombination in the subtelomeric region of the q arm between the marker GM354 (0 cM) and clcn5 (29 cM) and an abrupt increment of recombination between clcn5 (29 cM) and GM128 (77 cM) close to the centromere (Flpter=0.2). The unpairing region (20% of the total length) observed on the larger bivalents of XY fish during early pachytene in meiotic cells has been confirmed by DAPI staining and FISH to be at the terminal part of the q arm, opposite to the centromere.

Comparison with six other tilapia species (2n=44) revealed a well conserved karyological distribution of the suspected LGs associated with sex determination (1 and 3). Besides, in *O. karongae* (2n=38) it was shown by SATA and UNH995/UNH104 marker hybridisation that LG1 has been re-arranged into the subtelomeric chromosome 2 as a result of a telomere-telomere fusion.

A pool of 15 tilapia BAC clones previously localised on chromosome 1 and containing sex-linked AFLPs, dmrt1, dmrt4 and several SINEs were screened for new microsatellites; BACs were digested with SAU3AI and TC, GT, ATCT and CTGT probes radio-labelled with ^{32}P. The high abundance of repetitive sequences in the BACs used led to only one useful polymorphic and co-dominant marker being obtained, associated to a BAC clone containing a copy of the dmrt1 gene on chromosome 1 (Flpter=0.85).

Four linkage maps were constructed from an XY male, XY neofemale, XX neomale and XX female, mapping 4 and 8 markers on LG1 and LG3 (including the dmrt1 associated microsatellite) respectively. A specific sex-determination locus was identified on LG1 clearly linked with UNH995. However there appeared to be different allelic strengths for this sex determination locus, as shown by different sex ratios associated with different UNH995 genotypes. Additionally, one of the two XX fish mapped, showed the location of the recessive black blotching trait on LG3 (chromosome 1) between the markers GM128 and GM526, close to the centromere (Flpter=0.14).

The results presented suggest a nascent Y chromosome in early stage of differentiation in Nile tilapia and with a functional master gene on LG1 close to the marker UNH995 (Flpter=0.67) located on the q arm of a small submetacentric chromosome. The potential influences of the autosomal LG3 (chromosome 1) in sex differentiation are also discussed.

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1.1.1 Economic and social relevance

After rice, forest products, milk and wheat, fish are the fifth most important agriculture product and a major animal protein source available for humans. They provide 25% of the animal protein in developed countries and more than 75% in countries under development (FAO, 2003). Since the 1970s aquaculture activities have grown substantially, strongly contributing to the security of food supply through the world (Castillo, 2001). Tilapia species are the second group most important of fish after Chinese carps with over 2 million tonnes production per year (1.5 million tonnes from aquaculture and the rest wild caught) estimated for 2006, valued at almost US$3 billion (FAO, 2005).

According to FAO (2004), by the year 2015, tilapia production is expected to reach 5 million tonnes annually and will double again by 2030. In 2025, the total world human population will be 8.5 billion, requiring an estimated 55 million tonnes of aquatic food production coming mainly from aquaculture but complemented by fisheries. This projection includes a rise in the consumption of fish per capita from 16 kilos to 20 kilos per annum.

At present, imports of tilapia into the first world countries are growing rapidly because the mild white flesh of tilapia is readily accepted by consumers. For instance, the USA has recently experienced a boom in tilapia consumption, which has grown over 300% in the five past years (U.S. Foreign Trade Information, online access).

1.1.2 General features of cultured tilapia

The tilapiine tribe which is commonly named tilapia, included the genera *Sarotherodon*, *Oreochromis* and *Tilapia*, and the fourth genus, *Danakilia*, which comprises a single species (Sodsuk *et al.*, 1991, 1995). Cnaani *et al.* (2007) propose some tilapiine phylogenetic trees based on several allozymes and the ND2 mitocondrial gene respectively.

In this way, tilapia is a diverse tropical family with over 100 species originating from Africa and the Middle East. The most widespread and popular cultured tilapia species is the Nile Tilapia (*Oreochromis niloticus*) which contributes 85% of the total production (1.5 million tonnes/year), although the hybrid *O. niloticus* X *O. aureus* is included within this figure, and probably counts for most of the total tilapia production (Castillo, 2005). The remaining production (15%) comprises *O. mossambicus, O. aureus, O. andersoni, O. macrochir*, *O. spilurus, Sarotherodon galilaeus, S. melanotherodon, Tilapia rendalli, T. zillii, T. mariae*, the hybrids *O. mossambicus x O. urolepis hornorum* and other incompletely identified tilapia species and hybrids (tilapia nei) (FAO, 2004; Castillo, 2005).

Gonadal differentiation in tilapia species occurs in early stages, between 16 and 20 days after yolk absorption. The female gonads start to differentiate from seven to ten days before male gonads at temperatures over 24°C and with an optimum at 27°C (Secretaria de Pesca, 1993). Commercially exploited tilapias (mentioned above) reach sexual maturity after the third month of age, having a total length from 8 to 16 cm. The frequency of spawning depends on environmental factors, ranging from 6 to 16 times per year. Mating is stimulated by the same external factors that influence maturation such as temperature,

photoperiod, density of culture, food quality, etc. Mature males prepare a nesting area on the ground and defend its territory from other males. Then the female attracted into the male's territory spawns and the eggs are fertilised on the substrate or in the mouth depending on whether the specific species incubate the eggs externally such as *Tilapia spp.* (guarded nests) or orally (mouthbrooders) such as *Oreochromis and Sarotherodon spp.* (Bromage and Roberts, 1995).

In general, the standard time for each stage of development in *Oreochromis, Tilapia* and *Sarotherodon spp*. under optimum conditions (27°C) are from egg, 3-5 days; fry, 10-15 days; fingerling, 15-30 days; juvenile (7.0-12.0 cm), 45-60 days; and adult (10-18 cm), 70-90 days (Secretaria de Pesca, 1993).

The most relevant attributes of these species that makes them appropriate organisms for aquaculture are: 1. Great tolerance (plasticity) to changes in the culture environment such as oxygen and salinity; 2. Requires simple culture technologies; 3. Accepts a wide range of natural and artificial feeds; 4. Great tolerance to high densities and variations in culture temperature; 5. Relatively resistant to diseases; 6. Fast growth; and 7. One of the best advantages for genetic analysis is the short generational periods and flexibility in gamete manipulation (Bromage and Roberts, 1995).

Beside these points, tilapia flesh quality is accepted by consumers because of its firm texture, white colour and relatively soft intramuscular bones (Morales *et al.,* 1988).

1.1.3 Relevance of techniques for monosex culture

Intensive farming of tilapia mostly involves all male culture because of their faster growth and larger size compared to females, and also avoids the serious and widespread problem of early sexual maturation from females, unwanted reproduction, and overpopulation (Mair *et al.,* 1997) that can result in limited economical benefits (Mair *et al.,* 1995). Commercial techniques for all male tilapia production have been achieved traditionally by hand-sexing, hybridisation, hormone treatment and by the relatively recent development of genetically male tilapia (GMT) (Beardmore *et al.,* 2001).

Hand-sexing is regarded as being extremely labour intensive with a high risk of human error. Some interspecific crosses are known to produce all male tilapia hybrids such as the cross of a male *O. aureus* x female *O. niloticus*; however, this technique is not reliable unless the genetic purity of the parental stock is carefully maintained (Hulata *et al.,* 1995). In general, hormone treatment by the use of the typical synthetic androgen, 17-α-methyl testosterone (MT) has been proved to be the most effective, relatively inexpensive and practical method to masculinise fry of various tilapia species (e.g. *O. mossambicus, O. aureus, O. niloticus, T. zillii*) (Bromage and Roberts, 1995), achieving at least 95% male stock culture (Phelps and Pompma, 2000).

Genetic approaches have also become a key area of development for tilapia species in order to increase and improve their productivity. In this way, GMT production that consist in crosses between YY supermales and XX females to generate only XY male tilapia (for XX/XY systems such as *O. niloticus*, see Figure 1.1), has been proved to be the most sustainable technology for the long term and to represent at the moment the only option for

many countries (mainly developed countries) involving consumers and exporters where the new restrictions in the food additives legislation prohibit the use of hormones in animal products designated for human consumption (Roderick, 2007).

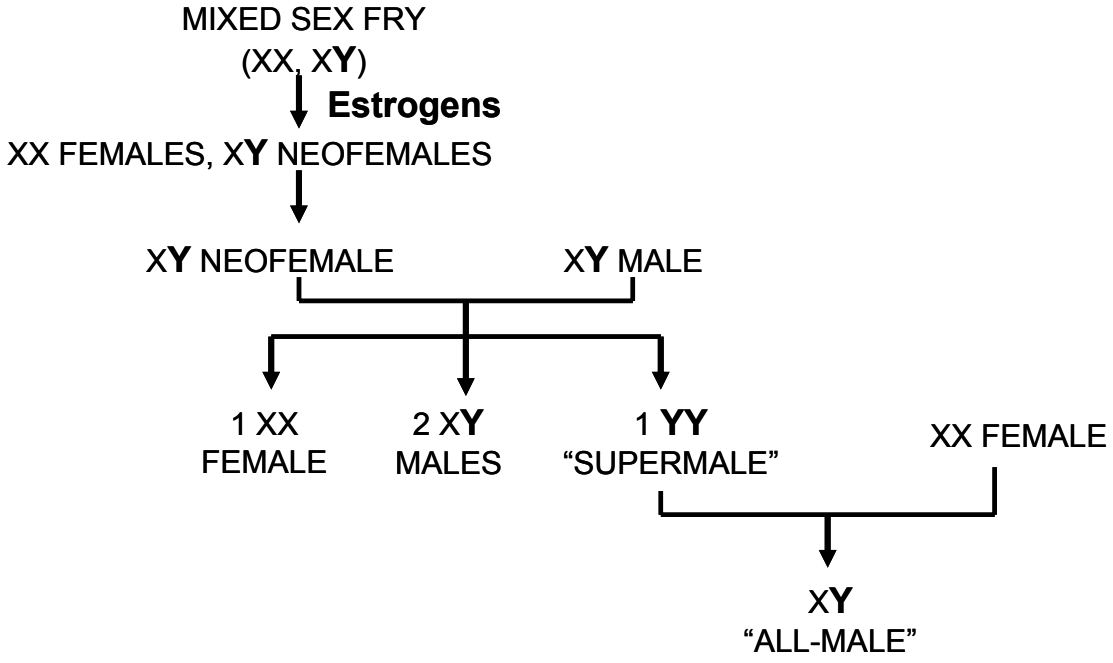

Figure 1.1 Hierarchical process commercially used for monosex male production or GMT in Nile tilapia *Oreochromis niloticus*. Extensive progeny-testing has to be done at every generation to ensure XY neofemales and YY supermales are statistically recognised. It is also required YY neofemales (sex reversing YY supermales with MT, not shown) for mass production.

Briefly, progeny-testing is defined as the process to identify testis and ovaries by acetocarmine staining (Guerrero and Shelton, 1974) of a given progeny. Males and females are then quantified and probability thresholds calculated according to Mair *et al.* (1994; 1997) criteria to define putative genotypes (XX, XY or YY) from parents.

Unfortunately, the meticulous process required to maintain and renew the YY broodstock together with several other genetic and environmental factors produce a range of 95-100% male progeny (Mair *et al.*, 1997). This aspect still represents obstacles to overcome before GMT technology could be declared to be the most popular method for commercial production of all male tilapia. Detailed study on the genetic mechanism that controls tilapia's sex determination (master sex determination gene, epistatic interactions, sex-linked markers, etc.) (Ezaz *et al.*, 2004b; Lee *et al.*, 2005; Shirak *et al.*, 2006; Cnaani *et al.*, In review), selection against autosomal effects on sex (Desprez *et al.*, 2003) and understanding of environmental influences on sex (Baroiller and D'cotta, 200; D'cotta *et al.,* 2001) represent the most plausible approaches.

1.2 Genome mapping

1.2.1 The relevance of genome mapping

Molecular biology has made possible the wide scale characterisation of genomes in all living organisms, first in humans, then in agriculture and now in aquaculture (Carvalho and Pitcher, 1995). Originally, the developed DNA-base genetic markers had the main purpose of improving fish stocks and strains for important quantitative traits loci (QTLs) such as faster growth and disease resistance (Carvalho and Pitcher, 1995) or for the evaluation of natural fish populations including behavioural, morphological, phylogeographic and other evolutionary studies (Martins *et al.,* 2004).

Genome mapping studies in fishes is a starting point to understand in more detail the functions of these genomes. Nowadays, more than 10 fish

species are in the process of being mapped (Danzmann and Gharbi, 2001); some of the more advanced gene maps in fish are for zebrafish (*Danio rerio*), pufferfishes (*Fugu rubripes* and *Tetraodon nigroviridis*) and medaka (*Oryzias latipes*) with most of the raw sequences available in public databases (e.g. zebrafish project: zfin.uoregon.edu and pufferfish project: fugu.hgmp.mrc.ac.uk).

A representative of the cichlid group, *O. niloticus*, has also been the subject of several advances in genome mapping but is far behind zebrafish and pufferfish. The Nile tilapia genome contains 1.06 gigabase pairs distributed over 22 chromosome pairs (Majumdar, 1986). There is a BAC-based physical map composed of 3,621 contigs (groups of aligned sequences) (Katagiri *et al.,* 2005) and a high density linkage map of 23 linkage groups spanning 1,311 cM (Lee *et al.,* 2005; Shirak *et al.,* 2006). The fingerprinting database is available in a public database at http://www.hcgs.unh.edu/cichlid/#bacs/, and the comparative linkage map at http://www.hcgs.unh.edu/comp/. In 2006, a sequencing project for Nile tilapia and other Cichlids from Lake Malawi was approved and currently the sequencing of the genomes is in progress by the Doe Joint Genome Institute Community Sequencing Program (JGI-CSP), USA, and with the scientific collaboration of the Cichlid Genome Consortium under the direction of Dr. Tomas D. Kocher. More information about this project can be found at http://hcgs.unh.edu/cichlid/TilapiaCSP2005.pdf.

Gene mapping is a hierarchical process utilising several molecular techniques to gain information on the genome. Genomes may be mapped at three different levels: 1. Physical maps that localises large DNA segments onto

the cytological karyotype of a species; 2. Genetic linkage map that assigns the linear order of DNA markers along the chromosomes based on recombination frequencies between loci; and 3. “Full” genome sequence characteristation by assembling raw DNA sequence reads (Danzmann and Gharbi, 2001). The construction and integration of the physical and linkage map for the Nile tilapia, represents at this moment the most practical approach to understand the structure and evolution of the Cichlid genome (Martins *et al.,* 2004).

1.2.2 Cytogenetic physical mapping

The introduction of Fluorescence *in situ* Hybridisation (FISH) techniques in the early 1980’s gave origin to “the colour revolution” (Durante, 2006). Early applications of FISH provided the opportunity to analyse symmetrically transmissible aberrations such as translocations, inversions, duplications, microdeletions and to identify chromosome changes that were 4Mb or slightly shorter in size without depending exclusively on the chromosome quality when compared with the traditional cytogenetic techniques such as G-banding pattern (Czepulkowski, 2001).

The FISH technique uses DNA probes to detect complementary DNA sequences along a chromosome with the target DNA that is still in its natural position, *in situ* (Tamarin, 2002). The probe is coupled with a fluorochrome which fluoresces when is exited by a particular wavelength of light emitted by a fluorescence microscope. A great advantage of this technique is the capacity to visualise several different probes at the same time by using different combination of fluorochromes (e.g. DAPI, blue; Propidium iodide, red; FITC,

green; rodhamine, red) and excitation wavelength (Henegariu *et al.,* 2001; Czepulkowski, 2001), being able to evaluate the molecular and cytogenetic information simultaneously.

For physical mapping purposes, the compact and linear arrangement of chromosomes during metaphase at mitosis or a more relaxed (less compacted) paired bivalents during the synaptonemal complex formation in pachytene at meiosis, represent the stages at which most cytogenetic analysis is focused, as the chromosomes are at a condensation stage that are easily recognised following staining treatments such as the traditional Giemsa or the fluorochrome DAPI staining (Phillips and Reed, 1996) mentioned above. Interphase cells also have been used on FISH techniques but with less relevance to physical mapping than metaphase and pachytene cells for its inaccuracy (Czepulkowski, 2001).

The different type of probes by origin are described as alpha satellite probes (repetitive DNA sequences mostly from the centromeric region of the chromosome), telomeric probes (repetitive DNA sequences found in the subtelomeric region of the chromosome), unique sequence probes (locus specific sequence that identify a specific chromosome region or gene) or chromosome paints (mixture of unique sequence probes from a single chromosome, usually from a flow-sorted chromosome-specific library such as Bacterial Artificial Chromosomes, BACs or Yeast Artificial Chromosmes, YACs) (Czepulkowski, 2001;Turner *et al.,* 2001b).

The genomes of several fish species have been partially physically mapped using repetitive sequences to localise centromeres, telomeres and sex

chromosomes (Martins *et al.,* 2004) as shown on *Sparus auratus*, *Oncorhynchus tshawytsha* and *Leporinus elongates* respectively (reviewed in Phillips and Reed, 1996). Another application is for the localisation of multicopy genes (rDNA and histones), providing valuable information for understanding their function and evolution in the genome as demonstrated in salmonids (Phillips and Reed, 1996). Chromosome microdissection was used to produce sex-chromosome-specific paint probes, revealing an homology arm to the Yq on a small autosome chromosome in lake trout (Phillips, 2001) and potential sequence differences demonstrated on cross hybridisation on the terminal part of q arm between the pair of larger chromosomes in Nile tilapia (Harvey *et al.,* 2002b). Full integration of the genetic linkage groups to chromosomes has been done recently in zebrafish and rainbow trout (Phillips *et al.,* 2006a; Phillips *et al.,* 2006b) using specific molecular markers contained in BAC clones as probes that were previously linked and subsequently identified on specific chromosomes.

To date, the physical map from *O. niloticus* is composed of major tandemly repeated DNAs (Martins *et al.,* 2004) such as satellite DNA SATA (230bp repeat sequence) located at the centromeric regions of the n=22 chromosomes (Franck *et al.,* 1994); satellite DNA SATB (1,900bp) found on the short arm of chromosome 4 (Franck and Wright, 1993); 18S rDNA found on chromosome 8, 10 and 15 (Martins *et al.,* 2000); 5S rDNA found on chromosome 3,9 and 13 (Martins *et al.,* 2002); telomeric repeats (TTAGGG), including two interstitial sequences on chromosome 1 (Chew *et al.,* 2002); and finally several short interspersed nuclear elements (SINEs, average 340bp) and a common long interspersed nuclear elements for cichlids (CiLINE2, 1,165bp)

(Oliveira *et al.,* 1999; Oliveira *et al.,* 2003), both distributed in small clusters through the genome but with higher concentrations at the termini of most chromosomes. This latest version of the physical map in *O. niloticus* represents a good basis for the addition of new markers (such as specific sequences identified on the linkage group map of tilapia; (Lee *et al.,* 2005).

1.2.3 Linkage mapping

During meiotic cell division, three of the most important events from the genetic point of view occur. During the initial phase of meiosis (prophase I), the "joining" of four units called tetrads take place and form chiasmata points to recombine genetic material (cross over) between homologous chromosomes. This process reshuffles the genes and creates new combinations of genes in the gametes, greatly increasing genetic and phenotypic variance in a population (Tamarin, 2002). The second important event is the reduction division from the diploid state (2n) to the haploid state (n), separating homologous chromosomes (one from mother and one from father, anaphase I) that form a pair randomly into the new cells, increasing again the genotypic and phenotypic variance; the reduction division and random separation refers to the law of segregation (first Mendel's law) and the law of independent assortment (second Mendel's law). The third important process during meiosis is the equational division where the replicated halves of each chromosome separate to create the gametocytes (anaphase II) or second polar body (released once the oocyte is fertilised) (Tave, 1993).

This brief description of the key events in meiosis leads to understanding the principles of genetic (linkage) mapping construction. The number of recombination events observed through the offspring's allele segregation between two markers determines the genetic distance of markers and genes on a linear arrangement by a given parent. One centimorgans (cM) represents a 1% chance of recombination but the physical distance that this represents varies among sexes, species and even the linkage group being examined (Danzmann and Gharbi, 2001). Physical maps have shown that 1cM in the human genome corresponds to one to two million base pairs (bp) or 1-2 mega base pairs (Mb), and demonstrated that linkage analysis can provide a first approximation of where genes are located in the genome and their association with higher resolution if compared with physical maps (Miesfield, 1999). Nevertheless, linkage mapping does not represent necessarily the actual physical distances among the loci due to evidence of specific variation in recombination rates along the genome, even when comparing the sexes of the same species (Nachman, 2002).

A genetic map construction based on information from the segregation of polymorphic markers (commonly Amplified Fragment Length Polymorphism, AFLPs; Random Amplified Polymorphic DNA; Microsatellites; or Single Nucleotide Polymorphism, SNPs) is divided into a four stage process. First, the genotypes from parents and progeny have to be scored in a source mapping family (e.g. gynogenetic haploids, inbred strain, outbred pedigree, mitotic gynogenetic or androgenetic diploids or gynogenetic half-tetrad diploids: reviewed in Danzmann and Gharbi (2001). Second, the segregation patterns of what allele and linkage are analysed for one of each contributing parent. Third,

the markers are separated into different linkage groups, and fourth the distances between markers are calculated using either the raw observed recombination levels (single crossover) or adjusted level if more than one crossover is expected per chromosome arm (Kosambi function assumes moderate amount of multiple crossing over; Haldane function assumes free crossing over) (Lynch and Walsh, 1998). Although the Kosambi function has been most often used for the generation of linear graphical maps, in fish and more specifically in teleosts, an overall low level of multiple crossovers has been observed in the range of 0% to 10% (Purdom, 1993). Moreover, there are some studies suggesting only one crossover event per bi-arm chromosome as observed in a comparative analysis on salmonids (Danzmann *et al.,* 2005); (Gharbi *et al.,* 2006) and also supported by the low intralocus variation in recombination rates observed in several gynogenetic diploid families of *O. niloticus* (Hussain *et al.,* 1994). Currently the use of Kosambi or Haldanes function in the construction of linkage maps in teleost is under debate.

Linkage maps have a wide range of applications. Some of them have been constructed to reveal the genetic architecture of divergent populations. For example, Rogers *et al.* (2007) demonstrated non complementary genomic regions associated with their sympatric speciation after their spatial separation between dwarfs and normal lake whitefish (*Coregonus clupeaformis*). Other linkage maps have been focused on the detection of genome rearrangements, association of functional genes or sex determination loci. This last example is specially characterised by the specific linkage differences between males and females by suppression of recombination around the sex determining locus in the heterozygous sex (Lorch, 2005), allowing the identification of sex-linked or

sex-specific markers. One of the ultimate goals for producing detailed linkage maps of each chromosome in aquaculture species is to provide a framework for Quantitative Trait Loci (QTL) analysis. This work can provide relevant information for the manipulation or isolation of genes controlling economically important traits specific for a single species or a group of species (Cnaani *et al.,* 2003; Liu and Cordes, 2004a; O'Malley *et al.,* 2003). Another final goal of linkage maps is the analysis of the genetic basis of phenotypic traits of not only commercially but also evolutionary and ecologically importance (Danzman, 2005).

1.3 Genetics of qualitative phenotypes in tilapia

1.3.1 Sex determination systems in Cichlids

Perciform fishes belong to an Order with more than 9,300 species (25% of all vertebrates). Within this Order, cichlids represent the most diverse group with over 3,000 species which dominates marine and freshwater habitats (Cichlid Genome Consortium, 2005). Cichlids located in the great lakes of East Africa have evolved in the very recent evolutionary past, i.e. within the last 10 million years (Kocher, 2004).

The developmental timing of gonadal differentiation varies depending on the expression of sexual lability of each species. In cichlids (including freshwater and marine water species) four general critical periods of sex determination and differentiation have been categorised during different life stages, at fertilisation (genetic sex determination), larva, juvenile and adult influenced, expressing lability for, environmental sex determination,

behaviourally-controlled sex determination and sequential hermaphroditism consecutively (Oldfield, 2005). These observations are supported generally in gonochoristic and hermaphroditic fish and specially in the early stages, between fertilisation and larva, by genotype (XY or WZ) - environment (temperature) interactions as observed in tilapia (Baroiller *et al.,* 1999). Moreover, genetic sex determination represents the first and probably the most important stage along the differentiation process in Cichlids (Oldfield, 2005).

Considering only genetic influences to understand the dynamics and evolution of the mentioned systems, recent research taking a simulated tilapia meta-population with random dispersal among demes but local mating within demes concluded that a dominant female mutant W gene in a population with male heterogamety (XY) has better chances to be fixed, and move to female heterogamety, than a comparable neutral mutation assuming an absence of individual selection (Vuilleumier *et al.,* 2007). In a larger population size, the fixation of the sex-determining mutant gene will take longer compared to a small population size. Starting from a single copy, the mean time for fixation of W in a deme of 1000 individuals was about 700 generations and this time considerably decreased if the deme was sub-divided into meta-populations or local populations. Such observations led to the hypothesis that male and female heterogamety may switch back and forth repeatedly within a lineage over relatively short evolutionary time (Ezaz *et al.,* 2006; Vuilleumier *et al.,* 2007), opening opportunities for their interaction with polymorphisms involving mate choice, speciation and chromosome evolution in teleost fishes but contrasting with the well conserved sex determination systems in birds and mammals (Mank *et al.,* 2006).

1.3.2 Sex linked genes

1.3.2.1 Sex chromosomes

The term Y-linked refers to loci found on Y chromosome, which control holandric traits (traits only found in males); loci found on both X and Y chromosomes are called pseudoautosomal (Tamarin, 2002) and sex-limited traits are those that are expressed in one sex despite the genes being present in both sexes. Sex-influenced or sex-conditioned refer to traits that are in both sexes but occur more in one sex than the other.

XY and WZ systems have been observed in fishes with six different variants (e.g. multiple W, X or Y chromosomes system, WXY system and X0 or Z0 system). Male heterogamety (XY system) is twice as common as female heterogamety (Devlin and Nagahama, 2002; Tave, 1993). Differentiated sex chromosomes XY or ZW has been demonstrated in Neotropic electric eel *Eigenmannia virescens* (Sternopygids), where the variation in the morphology of sex chromosomes (X_1X_2Y) had been observed by R-banding techniques and explaining sex chromosome differences by addition/deletion of heterochromatin (De Almeida-Toledo *et al.,* 2001b). In salmonids, the rainbow trout *Oncorhynchus mykiss* is another specific case in that differences between sex chromosomes (XY) were revealed by different brightness in DAPI staining (Phillips *et al.,* 2006b). However, these are the exceptions despite the monofactorial systems mentioned, presenting in most of the fish species studied, no heteromorphic sex chromosomes (Devlin and Nagahama, 2002). With the possible exception of *Geophagus brasiliensis* (Kornfield, 1984; in: (Oldfield, 2005), no cases of morphologically distinguishable sex chromosomes have been found in cichlids using traditional observation techniques.

In the simplified XY sex-determining system, the female carries identical sex chromosomes (XX) and the male a mismatched pair (XY) where the Y chromosome is unique for males (Ebeling and Chen, 1970) as demonstrated in *O. mossambicus* (Campos-Ramos *et al.,*, 2002) after SC pairing analysis. The heterogametic sex usually determines the sex of the offspring by a sex specific gene(s) that is contained on the Y chromosome. The opposite situation is observed in the WZ system, where the male carries the same set of chromosomes (ZZ) and the female a mismatched pair (WZ), the W chromosome being unique for females as observed in *O. aureus* (Guerrero, 1975). In this system the female determines the sex of the offspring.

Other variations in sex determination systems that have been described in other fish species include multiple sex chromosomes of the described XY or WZ systems. For instance, in a system with multiple X chromosomes, a female could present $X_1X_1X_2X_2$ and males X_1X_2Y chromosomes, where the heterogametic male still determines the sex of the offspring as it is the case of the filefish *Gobionellus shufeldti* (Pezold, 1984). In a multiple W chromosomes system, a female presents ZW_1W_2 and male ZZ chromosomes, where the heterogametic female has the capacity to determine the sex of offspring (Tave, 1993) such as the virolito *Apareiodon affinis* (Filho *et al.,* 1980). There are other cases where a WXY system is present such as in platyfish (*Xiphophorus maculatus*) (Kallman *et al.,* 1973). In this system, chromosome combinations of WY, WX and XX result in females and XY and YY result in males.

Among cichlids, Nile tilapia (*O. niloticus*) is one of the few species with recent and significant progress in sex chromosome identification. Differences

between the putative X and Y chromosome (the largest bivalents) has been detected by analysis of the synaptonemal complex (Carrasco *et al.,* 1999) and sequence differences observed in the long arm of the same chromosomes (Harvey *et al.,* 2002b). The observation of an accumulation of repetitive DNA sequences (transposable elements) in this same chromosomal region, detected by molecular-cytogenetic techniques (Harvey *et al.,* 2003b) has been interpreted to support this view. Moreover, (Oliveira and Wright, 1998) suggested that an early heterochromatinisation on this pair of chromosomes began 10 million years ago.

The fixation of sex-influencing genes in a population (motivated by the positive effects on the species) can be achieved by suppression of recombination of genetic material between homologous chromosomes (Griffin *et al.,* 2002); (Volff *et al.,* 2007). The specific factors affecting sex recombination in chromosomes are different according to the sex chromosome regions such as accumulation of repetitive sequences around the sex locus (de Almeida-Toledo and Foresti, 2001a), and different if compared with autosomes (Lorch, 2005; Winter *et al.,* 2002) such as decrease of association by a mutational process (Nachman, 2002). These recombination modifiers affecting sex determination genes can be specifically acting on sex factors in a gonosome of the heterogametic sex (Nei, 1969), limiting the synapsis between the same pair of chromosomes despite their great homology (e.g. 99% homology between X and Y in humans) (Tamarin, 2002).

Sex-specific genes have been discovered in relatively few species of fish. A male-determining gene, DMY/dmrt1bY, derived from the ancient dmrt1

(Kondo *et al.*, 2002) has been recently identified and sequenced in medaka (*Oryzias latipes*, Adrianichthyidae), a species without visually apparent sex chromosomes (Matsuda *et al.,* 2002; Nanda *et al.,* 2002). A male-specific DNA probe (OtY1) was isolated from chinook salmon (*Oncorhynchus tshawytscha*) by using a subtractive hybridisation technique and indirectly applied for the production of only females (Devlin *et al.,* 1994); and a male-specific growth hormone (GH) pseudogene also identified in chinook, coho, chum and pink salmon, and in some Japanese salmon species (Devlin and Nagahama, 2002; Devlin *et al.,* 2001; Zhang *et al.,* 2001);. Unfortunately, the gene(s) that govern sex determination in one species do(es) not necessarily have(has) the same hierarchy and/or function in the upstream path of sex differentiation in closely related species, as demonstrated in *Oryzias* species where the previously mentioned gene DMY/dmrt1bY (Volff *et al.,* 2007) from *O. latipes* has been inactivated in *O. luzonensis* (Kondo *et al.,* 2004) or is absent in *O. celebensis* (Kondo *et al.,* 2003). The same male-specific GH pseudogene found in some salmonids has not been identified in sockeye salmon, Atlantic salmon or rainbow trout (Devlin *et al.,* 2001). A final example is a DNA fragment that is a male sex-specific marker in the three-spined stickleback (*Gasterosteus aculeatus*) but non sex-specific in the nine-spined stickleback (*Pungitus pungitius*) (Griffiths *et al.,* 2000).

Following bulked segregant analysis in offspring from three single pair crosses of *O. niloticus* (XY, var. Stirling), a male sex-determining region was identified (associated with LG1), successfully predicting phenotype sex in 95% of progeny only in two of the crosses but with no association in the third one (Lee *et al.,* 2003). In the same *O. niloticus* var. Stirling, by looking for Amplified

Fragment Length Polymorphism (AFLP), three sex-linked markers (*OniX*420/*OniY*425, Oni382 and *OniY*227) were detected in a gynogenetic family, accurately predicting phenotypic sex close to 100% progeny, but only 83% in normal cross families (Ezaz *et al.,* 2004c). In another species, *O. aureus* (WZ), a female sex-determining region was also identified by linkage mapping. This had a strong association with LG3 but with an epistatic interaction to a locus on LG1 (close to the previously male determining-region proposed in *O. niloticus*) (Lee *et al.,* 2004). Additionally, hybrid crosses from these two species (*O.niloticus x O. aureus*), demonstrated a locus controlling sex-determination on LG3 (Lee *et al.,* 2005). Finally, by QTL analysis, an association with sex determination and sex specific mortality on LG23 has been demonstrated in the hybrid cross mentioned above (Shirak *et al.,* 2006). These particular situations in tilapia with different sex systems in close related species, illustrate a complex case where a potential sex chromosome in one species can be an autosomal in another and vice versa.

1.3.2.2 Autosomal influences on sex determination

It is of great relevance to determine whether a gene or genes are located on autosomes or sex chromosomes as their inheritance and fixation within the population will be influenced by this (Vuilleumier *et al.,* 2007). When a phenotype is controlled by two or more autosomal genes, a non-epistatic or epistatic interaction could lead to skewed sex ratios in the progeny as expected from a single master gene (Winter *et al.,* 2002). Epistatic interaction is described as the capacity of an allele at one locus to modify or suppress the phenotypic expression of an allele at another locus. These interactions are

subdivided in dominant epistasis, recessive epistasis, duplicate genes with cumulative effects, duplicate dominant gene interaction, and dominant and recessive interaction (Tave, 1993). In Cichlids, a clear epistatic interaction for sex determination has been described in *O. aureus* (WZ system) by linkage analysis, suggesting the action of a dominant allelic male repressor on LG3 (putative W chromosome) and a suppressed allelic male determiner on LG1 (putative Y chromosome in close related species) (Lee *et al.,* 2004). This was previously suggested by observations of 100% female progeny in hybrid crosses between heterogametic WZ females and XY males (Hulata *et al.,* 1995).

Widening the scope of factors that control sex in Cichlid species, it has been observed that sex not only could be controlled by sex chromosomes but also by other autosomal sex-influencing or sex modifying genes (Devlin and Nagahama, 2002). Autosomal genes (not linked with putative sex determining loci) with different levels of penetrance in sex determination may affect the production of 100% males in tilapia as proposed by Sarder *et al.* (1999) and Karayucel (1999).

Helping to understand the different degrees of expression from genes influencing sex determination in tilapia, it is commonly used in genetics two terms: penetrance and expressivity. Penetrance is the term used for the appearance in the phenotype of traits determined by the genotype. Most genotypes are fully penetrant, but not all are. Expressivity is a term that is also employed to describe the different grade of expression in the phenotype (Tamarin, 2002; Tave, 1993). In general, these two terms are commonly used

when a particular genotype does not produce the expected phenotype due to the influence of other gene(s).

In Nile tilapia (*O. niloticus*), there are at least two separate “sex reversal” loci that cause females to become males and at least one of these autosomal loci seems to be linked with the red body colour (Ezaz, 2002; Karayucel, 1999).

Autosomal sex-determination influence has also been described in salmonids such as Chinook salmon, a gonochoristic species, previously thought to have a strict sex determination control. Sex has not only been observed to be influenced by environmental factors but also by early autosomal sex reversals (0.8% penetrance) when comparing two populations with a common genetic origin (Metcalf and Gemmell, 2006).

1.3.2.3 Environmental effects on sex determination

The most common extrinsic environmental variable influencing sex in animals is temperature (Ezaz *et al.,* 2006), followed by pH, exogenous hormones, pollutants (Baroiller and D'cotta, 2001; Devlin and Nagahama, 2002) or even social interaction (Oldfield, 2005; Toguyeni *et al.,* 2002).

In fish, it is anticipated that environmental conditions have variable effects on sex determination depending on their genetic background and developmental stability of different strains. It has been demonstrated in tilapia species that sex determination is very labile. This depends on the exact combination of sex determining genes present in different species and strains. Environmental effects may be variable in strength and direction. Sex

determination may also be very sensitive to the level of inbreeding and as a result developmental stability within a strain (Abucay *et al.,* 1999; Purdom, 1993). It has been proposed that temperature lability may provide evolutionary advantages to tilapia species increasing the possibility for dispersal with a higher number of males. Temperature may also affect genetically the establishment of a master sex determination gene (on differentiated sex chromosomes) with complete control over the sex determination process, giving as a result the accumulation of balancing autosomal genetic factors (Devlin and Nagahama, 2002).

The adaptive nature of environmental influences on sex determination systems has been demonstrated unequivocally in at least one species, the silverside *Menidia menidia,* in which genotype-environment interaction was also shown (Conover and Kynard, 1981). Nevertheless, a stable genetic sex determination (GSD) still appears to operate in this species as demonstrated in medaka. Despite the discovery of the male-specific gene DMY/dmrt1bY (Matsuda *et al.,* 2002) in medaka, environmental effects on sex were demonstrated later with the temperature dependent sex determination of this species (Hattori *et al.,* 2007). It is also relevant to mention that genotype-environment interactions that determine gonadal fate in fish usually happen considerably earlier than histological differentiation (Oldfield, 2005) as demonstrated in medaka (Hattori *et al.,* 2007) and tilapia (D'cotta *et al.,* 2001).

1.3.3 Red body colour

Within the genera *Oreochromis,* strains and hybrids with red skin colour mutations have been developed, such as the single dominant red mutant in a pure strain of *O. niloticus* (McAndrew *et al.,* 1988) or the red hybrid *O. mossambicus* X *O. niloticus* (Jarimopas, 1988). These colour mutants have boosted the price and acceptance of tilapia. However, despite their improvements, mainly with the generation of hybrids, important disadvantages (such as less tolerant to changes in temperature and more susceptible to diseases and predation) in their culture have been recognised when comparing with normal coloured tilapia (Castillo, 2005), making their production more complex and expensive.

Due to a variety of tilapia species and hybrids giving different colour variants, the general term “red tilapia” is widely used for tilapia with a lack of the normal black pigmentation, that is characteristic of wild type fish (Hilsdorf *et al.,* 2002). For instance, a cross between *O. mossambicus* and *O. niloticus* gave rise to some of the first hybrid red tilapia using the dominant red colour mutation from *O. niloticus* (Jarimopas, 1988). This strain was called Taiwanese Red Tilapia (TRT). Other commercially important red species and hybrids include *O. mossambicus* Red Tilapia (MRT), *O. mossambicus x O. niloticus* Philippine Red Tilapia (PRT) (Avtalion and Reich, 1989), Florida Red Tilapia (FRT) (Desprez *et al.,* 2003) and the tetra-hybrid tilapia (males *O. niloticus x O. aureus* and females *O. mossambicus x O. hornorum*) Saint Peter strain (Souza *et al.,* 2000).

So the commercial interest in red tilapia and other colours have led to an increased interest in genetic studies. In this way, it has to be considered that

phenotypic expression depends on the number of genes and the way these genes act at each locus, assuming that all genes have more than one allele per locus, otherwise, no genetically heritable variation exists at that locus (Tave, 1993). Moreover, an observed phenotype could be due to a recessive loss-of-function mutation in a gene or a dominant gain-of-function mutation (Miesfeld, 1990).

The expression of each allele can lead to different phenotypes depending on the level of dominance (complete or incomplete) as illustrated in some cichlids. The dominant disorganised orange-blotch (OB) and the recessive wild type blue-black (BB) pattern (Streelman *et al.,* 2003) is an examples of complete dominance where the presence of one or two dominant alleles give the same phenotypic pattern.. On the contrary, in incomplete dominance the dominant allele is not strong enough to make the heterozygous phenotype identical to the homozygous dominant phenotype. *O. mossambicus* illustrates an example of how three different combinations of two alleles result in three different skin colorations resulting in black (GG), bronze (Gg) and gold (gg) (Tave, 1993).

As mentioned before, a single dominant red mutant (RR) has been observed in a pure strain of *O. niloticus*. Other colour mutations have been observed in Nile tilapia but with recessive expression and independent of red segregation such as blond and syrup (McAndrew *et al.,* 1988). In the genetic analysis of the red mutant, it has been observed that the red gene is acting as incomplete dominant in the presence of wild type, segregating according to Mendelian expectations. When a presumed heterozygous red with black

blotches (Rr) and wild type (rr) are crossed, the progeny shows a 1:1 ratio of red with black blotches : wild; crosses with a homozygous red (RR) and red with black blotches (Rr) give also a 1:1 ratio but of red : red with black blotches, and if the same red (RR) is crossed with wild type, it will give all red with blotches progeny (McAndrew *et al.,* 1988).

Karayucel *et al.* (2004) observed a red-linked locus with an effect on sex with low penetrance. Additionally, in a hybrid cross (*O.aureus x O. niloticus*) it has been demonstrated that red skin colour and sex-linked markers are located on linkage group 3 (Lee *et al.,* 2005). A major sex determination locus on LG3 has also been demonstrated in a pure species from *O. aureus* (Lee *et al.*, 2004). In this way, a deep study into the potential relationship (such as proximity and recombination events) between the red skin colour gene and putative sex determination on LG3 must be relevant in sex determination studies in tilapia.

1.4 Goal and Aims

The goal of this research was focused on obtaining a better understanding of the complex dynamics and evolution of the sex determination system in tilapia, mainly based on the observations from Nile tilapia *O. niloticus* and secondarily on other tilapia species; and to increase the knowledge of genes and markers closely involved in sex determination by comparison of physical and linkage maps.

To achieve this goal, the aims were focused on:

1. The development of an integrated and more accurate physical map of the putative sex chromosomes.

2. The development and identification of new markers associated to putative sex chromosomes.

3. The anchoring of new markers on linkage groups involved in sex determination and mapping of the existing sex related markers using tilapia families with different sex phenotype and genotype combinations.

4. The characterisation of sex chromosomes and sex linked regions by the integration of physical and linkage maps from Nile tilapia associated with sex determination.

5. The comparison of physical maps from the most representative sex-linked markers of Nile tilapia with other tilapia species such as *Oreochromis aureus, O. mossambicus, O. karongae, O. mortimeri, Sarotherodon galilaeus, Tilapia zillii* and *T. rendalli*.

1.5 Structure of the present thesis

The present study is divided in six chapters, of which three (Chapter 3, 4 and 5) describe in detail the fresh contribution of this research into the study of sex determination in tilapia.

The present Chapter (Chapter 1), general Introduction, gives the general background including basic concepts from which the present study was developed. Chapter 2, Materials and Methods, describes the relevant general laboratory techniques, giving as well as references for the biological material (tilapia resources) employed. Chapter 3, physical mapping, describes how some genes and DNA markers have been identified and physically mapped onto individual chromosomes by cytogenetic techniques (mitosis and meiosis), making relevant comparisons with previous developed physical and linkage maps and assigning some linkage groups onto chromosomes. Chapter 4, microsatellite isolation from chromosome 1, describes the whole process of microsatellite isolation from a pair of putative sex chromosomes, chromosome 1, using DNA from BAC clones mapped to it, aiming to anchor novel microsatellites on chromosome 1 for linkage mapping developed in the next chapter. Chapter 5, linkage mapping, is focused on the analysis of two relevant linkage groups associated with sex determination using several tilapia crosses (families) that combine different sex genotypes and phenotypes. The different linkage groups generated are compared and final comparisons are made considering physical maps findings from Chapter 3. Chapter 6, general discussion, covers important aspects to consider during the description and location of sex determination in tilapia, describes evolutionary points of view

with respect to XY/WZ systems interaction in this species and proposes further lines of research on sex determination in tilapia.

Chapter 2 GENERAL MATERIALS AND METHODS

2.1 Summary

The general methodology and techniques used throughout the study are described in this chapter. Some of the specific protocols and procedures are described in the relevant sections of the following chapters.

2.2 Experimental fish and basic fish handling techniques

2.2.1 Fish stock used

Eight tilapia species from three different genera were selected for this study for physical map comparisons, considering their differences in total chromosome number and/or availability at the University of Stirling fish stock: *Oreochromis niloticus, O. karongae, O. mossambicus, O. aureus, O. mortimeri, Tilapia zillii, T. rendalli and Sarotherodon galilaeus. O. niloticus* was used for most of the experiments; the rest of the species were used for comparative physical analysis (see Chapter 3) as they are closely related to the Nile tilapia, and more information is available on their genetics than the other tilapia species.

Tilapia taxonomy is recognised as follows (Trewavas, 1983) showing between brackets the genetic sex determining system;

Kingdom: Animalia

Phylum: Chordata

Subphylum: Vertebrata

Class: Actinopterylli (ray-finned fishes)

Order: Perciformes

Suborder: Labroidei

Family: Cichlidae (Ciclids)

Subfamily: Pseudocrenilabrinae

Genus: *Oreochromis*

Species:	*O. aureus*	(WZ)
	O. karongae	(WZ)
	O. mossambicus	(WZ / XY?)
	O. mortimeri	(XY?)
	O. niloticus	(XY)
Genus:	*Sarotherodon*	
Species:	*S. galilaeus*	(?)
Genus:	*Tilapia*	
Species:	*T. rendalli*	(?)
	T. zillii	(XY)

O. *niloticus*, *O. aureus, T. zillii and S. galilaeus* were introduced to the University of Stirling in 1979 (Majumdar, 1986; McAndrew *et al.,* 1988), originally from Lake Manzala, Egypt; *O. mossambicus, O. mortimeri* and *T. rendalli* from the Zambezi River, Zimbabwe introduced in 1985 (Majumdar and McAndrew, 1986; McAndrew, personal communication); and *O. karongae* from

Lake Malawi, Tanzania introduced in 1994 (McAndrew, personal communication).

All fish were monitored and handled according to the Working Procedures under the Animals (Scientific Procedures) Act 1986, monitored by the Home Office in the United Kingdom.

The starting mature broodstock of *O. niloticus* were selected according to phenotype (red or wild type skin colour; milt and egg production) and genotype (XX, XY and YY) required for the specific crosses and families described in Chapter 5. All broodstock and progeny in this study were photographed to provide an accurate record of colour segregation. Milt or eggs were stripped for visual gamete (phenotype) recognitions. Genotype was confirmed by progeny testing later on.

The starting XX and XY broodstock were produced by ordinary crosses of XX females x XY males. YY broodstock were produced as described in Chapter 5.1 with the cross of XY males x XY females.

2.2.2 Handling techniques

Female fish were individually stocked into glass tanks in a recirculation system in order to visually detect the swollen reddish genital papilla shortly before natural spawning. These ready-to-spawn females were anesthetised (by immersion in 0.01% ethyl-p-aminobenzoate, benzocaine; Cat. No. E1501, Sigma-Aldrich, UK; stock solution prepared at 10%, resuspended in absolute ethanol) and eggs released by slight ventral pressure. The eggs were collected

in clean glass beakers with clean warmed water (27°C) directly from the incubator system where the eggs were to be hatched. The sperm was collected from anaesthetised selected males in glass capillaries (1mm diameter) directly from the urogenital pore of the male once the urine was drained, by applying ventral pressure. This avoided activation of the sperm. Once the eggs were stripped and washed several times to remove any faeces, mucus and scales, they were left with enough water just to cover the eggs. Milt was added to the eggs in a proportion of approximately 100µl of milt per 1000 eggs and gently stirred for about 1 minute, then left standing for another 5 minutes to ensure maximum fertilisation rate before they were transferred into a plastic jar incubator in a recirculation system.

After the collection of milt and eggs, fish used were transferred into tanks with clean aerated water until complete recovered (10 min.). They were then returned to their original tanks.

Fertilised eggs were incubated for a total period of 12 days. Initial number of eggs, pigmentation rate, normal and abnormal embryos at hatching, and normal and abnormal fry before first external feeding were routinely recorded.

Fry were transferred into 25 l circular plastic tanks in a recirculation system immediately after the yolk sac was absorbed and reared for 4 months. At two months post hatch, fry densities were adjusted from 150 fish/tank down to 50 fish/tank to avoid any undesired effects on sex ratio from cannibalism and growth competition. For sex reversal purposes (see section 2.2.3 and 2.2.4 for sex reversion techniques), half of a batch of fry was transferred at the yolk sac reabsorption (YSR) into a static 25 l rectangular glass aquarium with a

thermostat to keep water temperature at 27°C, constant aeration and 20% water changes every two days. At the end of the fourth month, when fish had reached about 10 g total weight, fish were culled by immersion in an overdose of anaesthesia (0.05% benzocaine) and left immersed for at least 10 min before destruction of the brain and being sexed by gonad dissection. Fish that were kept for further crosses (such as F1 generation) were kept in similar 25 l tanks with densities of 25 fish/tank, which was found to be the minimum number of fish required to avoid mortalities through aggression.

When a mature fish was to be used in further crosses, the animal was marked with a 10 digit code TROVAN Passive Integrated Transponder (PIT) tag. For this procedure, fish were anesthetised as before, then a PIT-tag was introduced with the aid of a special wide tip syringe (previously disinfected in 70% ethanol) on the lateral-abdominal side of the fish, lifting a scale and making the incision under it. The incision was sealed with the same scale and fish was immediately placed into clean aerated water until full recovery before return to the original tank.

The other seven species of tilapia studied that came from ordinary crosses were reared similarly to *O. niloticus* and culled as required (phenotypic males and females). They were over anaesthetised as described before; blood removed for lymphocyte culture and then the brain was destroyed before gonad dissection.

2.2.3 Feeding techniques

Small fish from YSR to 3 g weight were fed with Trouw (UK) Ltd. Trout food no.2 and no.3 in a proportion 2:1, ground and sieved (<1 mm). Trout food no.2 had the composition 18% oil, 9% ash, 1% phosphorus, 54% protein, 1% fibre, +12000 iu/kg Vit.A, +2000 iu/kg Vit.D3, 250 iu/kg Vit.E. Trout Food no.3 (4.0 mm extruded pellets) had the composition 8% oil, 8% ash, 1.2% phosphorus, 40% protein, 2% fibre, 12000 iu/kg Vit.A, 2000 iu/kg Vit.D3, 100 iu/kg Vit.E. The frequency of feeding was three times per day (*ad libitum* according to the number and size of fish per batch).

Food for feminisation or masculinisation (ground and sieved trout food no.2 and no.3, proportion 2:1) was given to fry for a four week period after YSR in a static aquarium. Fish were fed three times a day *ad libitum* and any excess food was removed at the end of the day.

Fish from 3 g to 50 g weight were fed with Trout food no.2 only, as supplied by the manufacturer. The frequency of feeding was three times per day (*ad libitum* according to the number and size of fish per batch).

Adult fish (broodstock) of more than 50 g of weight, were fed with Trouw (UK) Ltd. Trout food no.3, supplied twice a day (*ad libitum* > 2% daily of body weight).

2.2.4 Steroid food preparation

To prepare 100 g of food for feminisation, the procedure according to Mair *et al.* (1994) was carried out with some modifications, as follows: 100 mg of

diethylstilbestrol (DES) hormone (Cat. No. D4628, SIGMA, UK) was dissolved in 30 ml absolute ethanol. Working within a fume hood, this stock solution was mixed with 100 g ground and sieved fish food (see section 2.2.3), gently pouring the stock solution onto the food and stirring with a spatula to homogenise the sample. The final concentration of hormone in the food was 1000 mg/kg. Once perfectly mixed, the treated food was allowed to dry before being packed into individual sealed plastic bags, stored at 4°C and protected from direct light. Treated food was kept for a maximum of three to four months to still ensure good working strength of the hormone.

To prepare 100 g of food for masculinisation, the procedure according to Abucay and Mair (1997) was carried out with some modifications, as follows: 30 mg of 17α-methyltestosterone (MT) hormone (Cat. No. M7252, SIGMA, UK) was diluted in 10ml of absolute ethanol (giving 3 mg/ml stock solution), then 1 ml of stock solution was diluted in 30 ml absolute ethanol. Using a fume hood, this diluted solution (the whole 30 ml) was mixed with 100 g ground and sieved fish food (see section 2.2.3), then dried and stored as described above. Final concentration was 30 mg/Kg.

2.2.5 Progeny-testing (physical sex determination)

In *O. niloticus*, after the offspring were culled as described above, a photograph of 10 to 15 randomly selected fish was taken for comparison purposes (skin colour, body shape and size). Skin colour patterns were recorded as presence or absence of black spots, all of them with pale red-skin colour background. The gonads of a maximum of 100 offspring per cross were

examined according to the methodology from (Guerrero and Shelton, 1974). Phenotypic sex was identified under a dissection microscope at 10× and 40× magnification, after adding one drop of 1% acetocarmine staining solution and making a squash of the whole gonad on a slide. Three different phenotypes were recorded: female (by the obvious presence of oocytes), male (by the presence of "porous" testicular tissue) or intersex (by the presence of both, oocytes and "porous" testicular tissue) as illustrated in Figure 2.1.

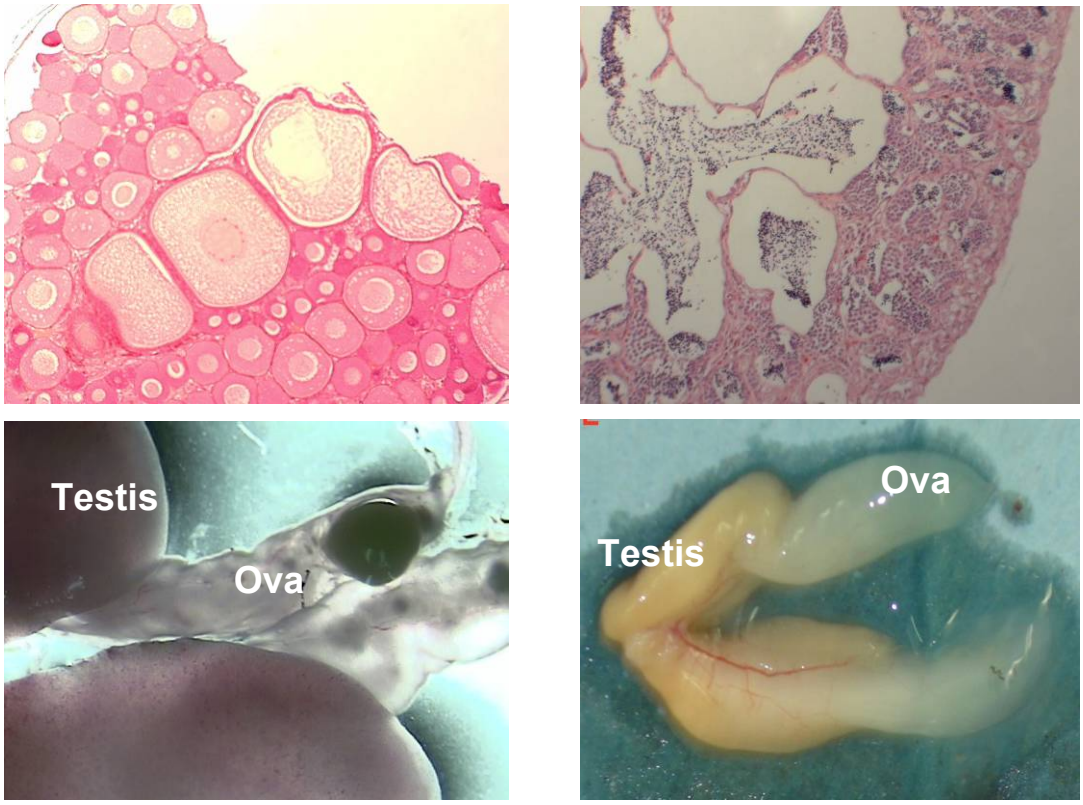

Figure 2.1. Pictures showing basic characteristics taken to differentiate ovaries, testes and ovotestes. a) Acetocarmine stain at 40× magnification; ovary with oocytes in different stages of maturation. b) Acetocarmine stain at 40× magnification; testes with porous tissue and sperm micro-ducts. c) and d) Fresh gonads at 10× and 1× magnification respectively; ovotestis having the anterior part developed as ova (translucid or whitish colour on the right of pictures) and posterior part with testicular tissue (pinkish or yellowish colour on the left of pictures), noticing the differences in shape and colour between both areas.

Finally, fin clips were taken from at least 50 sexed fish, preserved in 95% ethanol at room temperature and labelled for genotype analysis.

2.3 Tilapia Genomic DNA

2.3.1 Genomic DNA preparation

The only tissues used during this research for genomic DNA extraction were fin-clips preserved in 95% ethanol. Two DNA extraction techniques were used: the phenol-chloroform protocol of Taggart *et al.* (1992) with a few modifications, and the phenol-chloroform free PUREGENE DNA purification Kit (REAL laboratories, Spain).

The first technique was used for DNA extraction from the original broodstock, so the quantity and quality of these samples were enough to set up the molecular biology studies and work with them as controls for further optimisations of the protocols. The second technique was used for DNA extraction from the offspring, as the phenol-chloroform protocol is more complex, more time consuming and uses a highly toxic solution (phenol). The yield obtained from this alternative protocol was sufficient enough for the PCR analyses required, giving consistent results and allowing an efficient extraction of many more samples per unit time in comparison with the phenol-chloroform technique.

Phenol-chloroform extraction began with the digestion of approximately 10 mg of tissue in a 1.5 ml microcentrifuge tube with 375 µl solution containing 0.2M EDTA pH8.0, 0.5% SDS and 10 µl (20 mg/ml) proteinase K (SIGMA-Adrich, UK). Then, samples were digested overnight in an oven at 55°C. Next day, 1 µl (20 mg/ml) of RNAse (SIGMA-Aldrich, UK) was added for a further hour at 37°C. The RNAse free genomic DNA was then ready for phenol-chloroform extraction.

Working in a fume cupboard, an equal volume of phenol (pH 8 equilibrated) and then chloroform was added consecutively to the DNA solution. Samples were mixed by gentle inversion for 20 min after each addition and then centrifuged at 15,000 g for 5 min at room temperature (RT). The top aqueous phase containing the DNA (approximately 300 µl) was carefully transferred to a new tube using a 1ml wide end tip. These steps were repeated at least two times if a white precipitate was still observed between the aqueous and organic layers. Three volumes of 92% ethanol was added to the DNA solution for precipitation, mixed by gentle inversion and left for 3 min before centrifuging at 15,000 g for 5 min at RT. The liquid was carefully decanted and 1ml of 70% ethanol added; after 30 min with gentle rotation the sample was again centrifuged as before and the ethanol decanted, then any ethanol remaining was removed with a micropipette. The sample was air dried and resuspended in 50 µl of resuspension solution (proportions 1:3 of 0.25× TE buffer, pH8.0, and molecular grade water) then left for at least one day at RT for complete resuspension (see Appendix I for TE buffer preparation).

The PUREGENE DNA purification Kit (protocol for genomic DNA extraction from animal tissues) was scaled down to 100 µl per reaction with several modifications for the use of low profile 96 well plates (200 µl capacity) with individual strip caps. One biopsy punch of approximately 3 mm^2 was digested in 100 µl proprietary lysis solution with 0.5 µl of proteinase K (20 mg/ml stock solution) by incubating overnight at 55°C. The next day 60 µl of protein precipitator solution at RT was added and left standing for about 15 min, rotating gently several times at intervals. Precipitated proteins were centrifuged at 3,000 g for 20 min, and then 150 µl of supernatant was removed into a new

well plate and 100 µl absolute isopropanol was added. The sample plate was rotated gently aiming to see the precipitated DNA after several mixings. The plate was then centrifuged again at 3,000 g for 30 sec which was sufficient time to pellet the DNA to the bottom of the well. With some practice, the isopropanol was poured from the whole plate shaking it twice while facing down and then any excess isopropanol was dried by leaving the plate facing down on a clean sheet of tissue paper. Samples were washed with 150 µl of 70% ethanol by inverting slowly for 5 min, spun down at 3,000 g for 5 min and the ethanol removed (any ethanol remaining after pouring it out was removed with a 10 µl micropipette) and left until complete dry. Samples were resuspended in 40 µl of hydration solution (provided with the kit). Resuspension was assisted by heating to 65°C for 30 min and then at RT overnight.

2.3.2 Genomic DNA quantification

Accurate DNA quantification was done by spectrophotometry (nanodrop ND-1000). All genomic DNA samples were quantified this way before being used for further procedures. DNA concentrations were standardised to between 50 to 100 ng/µl by adjusting with molecular grade water. DNA purity as measured by OD_{260}/OD_{280} fell between 1.7 - 1.9.

Further quantitative and qualitative analyses were also carried out by electrophoresis for some critical samples (e.g. DNA to be used in ligation techniques, see below). A 1.0-1.5% agarose gel was prepared containing ethidium bromide (0.5 µg/ml) for UV light visualisation. The electrophoresis was run at .70-10 V/cm in 1× TAE buffer (see Appendix I) for about an hour. Size of

fragments and approximate amount of DNA was estimated by calibrating DNA concentration against known size standards, such as λDNA/HindIII digested DNA (see Appendix III). Migration distances were compared for specific size determination among the unknown fragments and the standard ladders. Genomic DNA was expected to have a high molecular weight with a single band present above 23 Kb and should not show the presence of a significant low molecular weight DNA (degraded).

2.3.3 DNA amplification standard conditions (PCR)

The Polymerase Chain Reaction (PCR) standard conditions using genomic DNA or plasmid DNA as templates are presented below (Table 2.1 and Table 2.2). PCR reactions were routinely performed in 0.2 ml DNAse free thin-walled microtubes or plates. A commercial ABgene™ Taq DNA polymerase kit (UK) was used; included 10× Buffer IV (750 mMTris-HCl pH 8.8, 200 mM $(NH_4)_2SO_4$ and 0.1% (v/v) Tween20), 25 mM $MgCl_2$ and 5 U/µl Taq polymerase. Designed primers (18-27 bases) were ordered from MWG (Germany) and initially resuspended in molecular grade water to a standard stock concentration of 100 pmol/µl (100 µM).

PCR products were checked on 1.5% agarose gel depending on the expected amplicon sizes. Other standard conditions for electrophoresis runs were as mentioned in section 2.3.2.

Table 2.1 Standard PCR components for amplification of genomic DNA and constructs.

Components	Stock Solutions	Per Reaction	PCR volumes (µl)
Molecular Grade Water	---	---	8.11-8.71
Buffer IV	10×	1×	1.50
$MgCl_2$	25 mM	1.5-2.5 mM*	0.90-1.5
dNTPs	5 mM each**	0.15 mM	0.45
Primer F	10 µM	1 µM	1.50
Primer R	10 µM	1 µM	1.50
Taq Polymerase	5 U/µl	0.2 U	0.04
Template DNA	100 ng/µl	100 ng	1.00
TOTAL	---	---	**15.00**

*** Concentration of $MgCl_2$ was optimised within this range according to the amplification performance.**
**** dNTPs were prepared in a previous master mix using equal amounts of dATP, dCTP, dGTP and dTTP (5/5/5/5) making a total solution stock of 20mM.**

Table 2.2 Standard PCR cycle parameters for amplification of genomic DNA.

Steps	Cycle Parameters		
Initial Denaturation	1 cycle	96°C	2 minutes
Denaturation Annealing Extension	25 cycles	94°C 55-60°C* 72°C	50 seconds 30 seconds 50 seconds
Final Extension	1 cycle	72°C	7 minutes
Hold	---	4°C	---

*** Annealing temperature was optimised within this range according to the amplification performance.**

2.4 DNA cloning

2.4.1 Isolation of BAC construct DNA from library clones

First, selected BAC clones from the Tilapia BAC library were picked with a sterile toothpick and inoculated into 500 µl of Miller LB-broth (MERCK, Germany) containing chloramphenicol (12.5 µg/ml), Approximately 50µl of this inoculated medium was poured onto a sterile 27 cm^2 plastic petri dish containing Miller LB-agar (MERCK, Germany) / chloramphenicol (12.5 µg/ml), using a glass rod to spread the liquid medium with cells over the agar surface. Inoculated agar medium was incubated overnight at 37°C. The following day, a single colony was selected with a toothpick and grown overnight at 37°C in a loose-cap tube with 5 ml of LB-broth / chloramphenicol. A second growing stage was required to increase the volume of the cell culture medium, this time inoculating 30 ml of LB-broth / chloramphenicol in a sterile plastic loose-cap tube with 60 µl of the overnight cell culture (1/500). This cell culture was incubated overnight as before.

After the 15 h, the expected cell concentration was about $2\text{-}4\times10^9$ cells/ml (OD_{600} = 0.2-0.4), in early stationary phase. Approx. 500 µl of this culture was preserved as a BAC clone stock. This was transferred into a 1.5 ml microcentrifuge tube and centrifuged at 1000 g for 5 min. The growth medium was replaced with fresh medium including 15% sterile glycerol, cells were resuspended and the tubes stored at -80°C.

The remainder of the cell suspension was used for plasmid extraction. Due to cost considerations a part proprietory extraction procedures was employed. Cells were concentrated by centrifugation at 2,500 g for 10 min at

4°C. Supernatant was discarded before adding 1.5 ml of Qiagen® Solution 1 containing RNase cocktail, then vortexed to resuspend the cells. Once there were no visible clumps of cells, 1.5 ml of solution 2 was added, mixing by slow inversion for 1 min and left at RT for 2 min. Next, 1.5 ml of solution 3 was added, mixing by slow inversion for 1 min and left at RT for 3 min. The sample was then poured onto a 0.45 µm Fisher-Nalgene® sterilisation filter (Cat. No. 245-0045) and vacuum source was applied until the sample had been drawn through. The filtrate was transferred into several clean 1.5 ml microcentrifuge tubes (0.8 ml of sample each) and 0.56 ml of RT isopropanol was added to each tube. Tubes were inverted gently for about five min. They were centrifuged at 21,000 g for 30 min. The supernatant discarded and pellets washed with 1 ml of 70% ethanol, centrifuged at 21,000 g for 15 min and the supernatant discarded. Samples were vacuum-dried in a DNA speed VAC (Savant, DNA110 model) until no visible liquid remained. Resolubilisation was in 20 µl of 0.25× TE. Samples were left on the bench at RT for two days and vortexed before quantification. Plasmid sizes expected were in the range between 100-300kb (Katagiri *et al.*, 2005). Total yields were between 16 to 20 µg from 30 ml cell culture and concentrations standardised to between 50-100 ng/µl.

2.4.2 Subcloning of BAC construct fragments

*Sau*3AI restriction enzyme (New England BioLabs, UK) that generates cohesive -GATC- ends was used for BAC construct digestion (Table 2.3). Using 0.2 ml microcentrifuge tubes, incubation was at 37°C overnight. Enzyme

inactivation was achieved by incubation at 80°C for 20 min. Fragment size was then checked to confirm total digestion by electrophoresis, loading 1 µl on a 1.5% agarose gel.

Table 2.3 Composition used for digestion of BAC clone fragments, previous to ligation.

Component	Stock Solution	Volumes (µl)
Molecular Grade water	---	39.5
Plasmid DNA	1 µg/µl	1.0
Buffer *Sau*3AI	10×	5.0
*Sau*3AI	4 U/µl	4.0
BSA (25 mg/ml)	100×	0.5
TOTAL	---	**50.0**

Using 0.8% low melting agarose gel containing ethidium bromide (0.5 µg/ml), 13 µl of the digested sample was loaded in a single large well. A 100 bp ladder was run beside the sample to identify fragment sizes between 200-700 bp. The electrophoresis was run at 0.7-10 V/cm for 15-20 min. The targeted fragment sizes were cut from the gel with a clean scalpel blade and extracted with a Concert Rapid gel extraction system kit (Life technologies, Gibco BRL, UK) following the manufacturer's instructions. After recovery, the target fragments were resuspended in molecular grade water, DNA concentration was measured with a spectrophotometer and adjusted to 10 ng/µl. Construct DNA fragments were then ready for ligation procedures.

The vector used for subcloning was pBluescript® II KS- (3.0 Kb) containing ampicillin resistance, multiple cloning sites and lac promoter (Stratagene, USA);

see Appendix IV). It was digested with *Bam*HI restriction enzyme (England Biolabs, UK), generating cohesive -GATC- ends (Table 2.4).

Table 2.4 Composition used for digestion of the pBluescript vector, previous to ligation.

Component	Stock Solution	Volumes (µl)
Molecular Grade Water	---	14.8
pBluescript II KS-	1.1 µg/µl	1.0
Buffer II	10×	2.0
*Bam*HI	20 U/µl	2.0
BSA (25 mg/ml)	100×	0.2
TOTAL	---	**20.0**

Using 0.2 ml microcentrifuge tubes, incubation was carried out at 37°C overnight. The enzyme was then inactivated by incubation at 80°C for 20 min. Complete digestion of template was checked by electrophoresis of 1 µl on a 1.5% agarose gel.

Before the vector was used for ligation procedures, the cohesive ends were dephosphorylated. To calculate the amount of Shrimp Alkaline Phosphatase (SAP; Boehringer Mannheim Laboratories, Roche, Switzerland) required, the following formula was applied based on the characteristics of the vector to dephosphorylate:

(Conc.vector in µg / size vector in kb) × 3.04 = pmol of ends

Then, up to 1 pmol of 5' terminal phosphorylated vector fragments (either 5' protruding or recessive ends) were incubated with 1 unit of SAP at 37°C for

10 min. The composition of the mixture was: 7 µl of vector (50 ng/µl), 0.9 µl of the 10× Buffer supplied (660 mM Tris-HCl, 50 mM $MgCl_2$, pH 8.5 at 20°C) and 1 µl of SAP (1 U/µl) making a total volume of 9 µl. After the incubation period, the enzyme was inactivated by heating the mixture at 65°C for 15 min. Electrophoresis of 2 µl of the sample was used then to confirm the expected 3 kb size of the open vector (already cut and dephosphorylated). Further phenol-chloroform DNA extraction was run for purification and concentration of the vector before its use in ligation procedures.

For ligation purposes, three different molar ratios of Vector:Insert were investigated (3:1, 1:1, 1:3). The starting concentrations were 50 ng/µl for the vector solution and 10 ng/µl for the insert solution. Working on ice, T4 DNA Ligase kit (100 U/µl, Cat. No. 481-220; ROCHE, Germany) was used for ligation, following the recommendations from the manufacturer. Each proportion of vector:insert was mixed in a 10 µl final volume consisting of 1× ligation buffer (660 mM Tris-HCl, 50 mM $MgCl_2$, 50 mM DTT, 10 mM ATP, pH 7.5 at 20°C), T4 DNA Ligase and molecular grade water, incubating at 14°C overnight.

2.4.3 Transformation

After overnight ligation, Super Competent Cells, SCS1® (Promega, UK) were used for transformation following the standard procedure described by the manufacturer. In a 1.5 ml polypropylene Falcon tube, 100 µl of super competent cells (thawed on ice 15 min before) and 1.7 µl of β-mercaptoethanol were added, and incubated in ice for 10 min, swirling gently every 2 min. 1 µl (10 ng/µl) of the ligation product was added, swirled gently and incubated in ice for

30 min. The Falcon tube was heat pulsed at 42°C for 30 sec and incubated in ice for 2 min. 0.9ml of preheated SOC medium was added (see Appendix I). After this, the sample was incubated and gently shaken (100 g) for a further hour at 37°C.

2.4.4 Blue-white colony screening and PCR analysis

LB-agar plates were prepared in advance before the transformation procedure was done in order to use the transformed cells immediately after the last incubation.

The 27 cm^2 LB-agar plates contained 80 µg/ml of methicillin (Cat. No. M-6535; SIGMA, UK) and 20 µg/ml of ampicillin (Cat. No. A-9393; SIGMA, UK) for selection of recombinant bacteria and suppress satellite colonies formation. 80 µl of X-gal (20 µg/ml stock solution diluted in Dimethyl Formamide, DMF; Cat. No. 15520-034, GIBCO, UK) and 40 µl of Isopropyl β-D-thiogalactogyranoside (IPTG, 100 mM stock solution diluted in molecular grade water; Cat. No. 15529-019, GIBCO, UK) were added onto the surface of the agar plate to enable standard white / blue colonies selection (Lac-2 based colour selection), corresponding to presence and absence of DNA fragment inserts into the plasmid, respectively.

The next step was to take 10 µl of transformed cell solution and mix them with 20 µl SOC medium. The mixture was then spread over the selective plate (prewarmed to 37°C) and plates incubated overnight at 37°C.

The total number of white and blue colonies were counted to estimate the transformation efficiency rate for the 3 vector:insert molar ratios used. To

enhance potential development of blue colour, plates with colonies were put into a fridge for about 4 h, before counting.

To check whether the white colonies had an expected sized insert, white, blue and weak-blue colonies were selected from a single plate and inoculated into 100 µl of LB-broth + both antibiotics in a 200 µl microcentrifuge tube. The cultures were grown for a further 4 h and then centrifuged at 1000 g for 10 min and the medium was substituted with TE buffer before resuspending the cells again. This resuspension was heated to 95°C for 5 min (to lyse the cell membranes), then cooled immediately to 4°C. The resultant cell debris was spun down at 10,000 g for 5 min and then the samples were returned to ice. Finally, the supernatant was transferred into a new tube and 2 µl of this was used for PCR using M13 Universal primers (M13F 5'-TCACACAGGAAACA GCTATGAC-3', and M13R 5'-ACGTTGTAAAACGACGGCCAG-3') that flank the insert (following the standard PCR master mix composition; see section 2.3.3). The PCR cycling conditions used are shown in Table 2.5.

PCR products were checked on 1.5% agarose gel. Each insert size was calculated as amplicon size minus the known flanking vector sequence size (220 bp).

Once the presence of inserts were corroborated and the number of colonies per cm^2 was estimated from a single transformation reaction, colonies were grown on a larger scale on a 500 cm^2 petri dish for the final screen of positive colonies.

Table 2.5 PCR cycling conditions used for amplification of transformed BAC fragments.

Steps	Cycle Parameters		
Initial Denaturation	1 cycle	95°C	5 minutes
Denaturation Annealing Extension	3 cycles	95°C 50°C 72°C	1 minute 1.3 minutes 1.3 minutes
Denaturation Annealing Extension	25 cycles	95°C 50°C 72°C	30 seconds 30 seconds 60 seconds
Final Extension	1 cycle	72°C	7 minutes
Hold	---	4°C	---

2.4.5 Design and radio-labelling of probes

For screening of the white colonies previously selected, four of the most common microsatellite repeat motifs reported in tilapia (NCBI / GenBank sequences) were used. These were combined in a single probe preparation as detailed in Table 2.6 (Oligos were ordered from MWG, Germany).

The probe mix was 5' radioisotope labelled by standard techniques as outlined in Table 2.7. The mixture was incubated for 30 min at 37°C in a water bath. Finally, denaturation and inactivation of the enzyme was performed for 20 min at 95°C and then sample centrifuged briefly before use.

Table 2.6 Probes developed for the screening of BACs from tilapia after label them with the isotope γ ^{32}P-ATP.

Repetitive Probe	Length (bp)	Tm °C	Molar proportion used
(GT)11	22	60.3	1
(GA)11	22	60.3	1
(GACA)5-GA	22	60.3	1
(GATA)7	28	56.3	1

Table 2.7 Formula used for radiolabelling (γ ^{32}P-ATP) of oligo probes.

Component	Stock solution	Volumes (µl)
Molecular Grade Water	---	6.5
Oligo mixture *	10 pmol/µl	1.0
gamma ^{32}P-ATP **	2.2 pmol/µl	1.5
T4 Buffer	10×	1.0
T4 polynucleotide kinase ***	10 U/µl	1.0
TOTAL	---	**11.0**

*** See Table 2.6.**
**** Specific activity 4,500 Ci/mmol; 10 µCi/µl; ICN/Biomedicals, USA.**
***** Supplied by ROCHE, Germany.**

2.4.6 Identification and isolation of putative microsatellite markers

Ideally, the identification and isolation of positive clones should be completed within three days of plating the bacteria, to avoid contamination issues. For the preparation of solutions used in this section, see Appendix I.

Standard colony lifts were performed using Hybond™-N+ membranes (Amersham Pharmacia Bioscience, UK) according to manufacturer protocol. The membrane with colonies was placed face down onto a new LB-agar + antibiotics dish for a further three hours (in order to replicate template). After this incubation period, the membrane was placed colony side up onto a series of four petri dishes with filter papers soaked in different solutions: 1) Lysis solution (10% Sodium Lauryl Sulfate; SDS) for 5 min; 2) Denaturing solution (0.4 M NaOH and 0.6 M NaCl) for 5 min; 3) neutralising solution (0.5 M Tris-HCl and 1.5 M NaCl) for 5 min; and 4) Conditioning and digesting solution (2× Sodium Saline Citrate; SSC) with 2 mg/ml proteinase K for 15 min. Excess liquid between treatments was absorbed by placing the membrane on a clean tissue paper. The next step was to place the membrane between two filter papers and scrape it with a ruler to remove any remaining cellular debris. Finally the membrane was baked at 80°C for 2 h and stored at RT wrapped in plastic film to avoid any contamination.

When the radio-labelled probes were prepared, the membrane was soaked and washed in 0.1% SDS solution at 84°C for 5 min, using soaked tissue paper and manually scrubbing the surface of the membrane to remove any excess of cellular debris.

Hybridisation was carried overnight in a hybridising oven, Hybridiser HB-1 (Techne, UK). This oven is equipped with removable glass cylinders, and allows constant rotation during the hybridisation period. Church's solution (Appendix I) was used for both pre-hybridisation (25 ml, incubation for 3.3 h at 60°C) and hybridisation (15 ml, incubation overnight at 53°C). The membranes

were placed into the cylinder face-in and 11 µl of probe solution (13 pmol in total) were added for the hybridisation step.

Post-washing steps were developed at low stringency because of the small size of probes. Membranes were washed two times in 100 ml of 6× SSC + 0.2% SDS and three times in 100 ml each of 5× SSC + 0.2% SDS. All washes were at 53°C for 5 min. A last wash was using 7× SSC. After this, membranes were placed on tissue paper (colony side up) to remove any excess liquid and then wrapped individually with plastic film.

Working in a dark room, the membrane was placed into an autoradiograph cassette (with intensifying screens) for overnight autoradiograph exposure at -70°C. Next day the autoradiograph (KODAK BioMax Light; Eastman Kodak company, UK) was developed under red-safelight conditions (KODAK GBX-2 safelight filter). Two trays larger than the sheet film to be processed were prepared, containing diluted developer (KODAK GBX Developer and Replenisher) and fixer (KODAK GBX Fixer and Replenisher) respectively. The film was placed for 5min in each solution with a quick rinse of water before transfer to the fixer, and finally rinsed again in running water for 5 min; this was then hung up with a clip attached to one corner and left until completely dry.

Positive dark spots on the autoradiograph were matched with the original colony dish and selected colonies picked up with sterile toothpicks to be transferred onto a small selective LB-agar petri dish (27 cm^2) with X-gal and IPTG reagents for blue/white differentiation. Several colonies were streaked in a radial way onto the dish. After overnight incubation at 37°C, true white colonies were picked up again and inoculated into 96 U-bottom well plates each

containing 60 µl of LB-broth + antibiotics. Incubation was at 37°C for 2 h and then overnight at RT. Next day the growth medium was changed following centrifugation and replaced with fresh medium containing 15% of glycerol. Before storing these at -70°C as backup, individual colonies were grown in 5 ml LB-broth + antibiotics as described in section 2.4.7 for plasmid preparations.

2.4.7 Plasmid DNA preparation

Plasmid preparation was performed from selected positive clones (containing constructs) using the Qiagen® Plasmid Mini kit (Qiagen, UK) protocol according to the manufacturer with some modifications. This plasmid preparation kit was specially used for plasmids up to 50 Kb. Buffers P1, P2, P3, QBT, QC and QF together with Qiagen-tips was provided with the kit.

After growing cell cultures overnight (15 h), inoculated in 5 ml LB-broth + methicillin (80 µg/ml) and ampicillin (20 µg/ml) medium, cells were spun down for at 1,000 g for 5 min, supernatant removed and resuspended in 600 µl of Buffer P1 with gentle vortexing. Then 600 µl of buffer P2 was added, mixing gently and with incubation for 5 min at RT, followed by the addition of chilled Buffer P3 and incubation on ice for 5 min. After the last incubation, the sample was centrifuged at 14,000 g for 15 min, producing a clear supernatant with a white pellet (precipitated protein) at the bottom. While centrifuging the sample, Qiagen columns (one per sample) were equilibrated with buffer QBT, by adding 1 ml to top of column and allowing draining by gravity flow. At this point, supernatant containing the construct sample was applied to the column, which was washed four times with 1 ml Buffer QC.

Constructs were eluted from the columns with 800 µl Buffer QF heated at 65°C and recovered into a 1.5 ml microcentrifuge tube. Then constructs were precipitated with 0.7 volumes of isopropanol, centrifuged immediately at 14,000 g for 30 min and the supernatant was decanted. Pellets were then washed with 1 ml of 70% ethanol, and samples air dried until there was no evidence of ethanol. Final resuspension was done in 20 µl of 0.25× TE and left on the bench for a day at RT before quantification and storage at -20°C.

2.5 DNA Sequencing

2.5.1 Sequencing on ABI Prism 377 and data analysis

Approximately 300 ng plasmid were used for cycle-sequencing reactions following the manufacturer's protocol for DYEnamic™ ET Terminator (Amersham Pharmacia Biotech, England) as described in Table 2.8 and Table 2.9. The sequencing primer employed were vector specific M13 forward and reverse, and both strands of insert DNA were single-pass sequenced. Reactions were prepared in 0.2 ml microcentrifuge tubes.

Table 2.8 Components used for cycle-sequencing reactions. Notice that forward and reverse primers were used in separate reactions with the same plasmid DNA.

Components	Stock Solutions	Volumes (µl)
Molecular Grade Water	---	0.9
Sequencing premix	1×	1.6
M13 forward or reverse primer	10 pmol/µl	0.5
Plasmid DNA	150 ng/µl	2.0
TOTAL	---	**5.0**

Table 2.9 Programme parameters for cycle-sequencing reactions.

Steps	Cycle Parameters		
Denaturation Annealing Extension	25 cycles	95°C 50°C 72°C	20 seconds 15 seconds 60 seconds
Hold	---	10°C	---

Following the cycle sequencing reaction, 5 µl of 3 M Sodium Acetate and 20 µl of 95% ethanol were added to the 5µl sample, mixed briefly and left at RT for 15 min. Samples were centrifuged at 20,000 g for 20 min. The supernatant was removed very carefully with 200 µl micropipettes avoiding touching the pellets, then 180 µl of 70% ethanol was added and mixed thoroughly. A second centrifugation step was performed at 20,000 g for 5 min. After carefully removing the supernatant, the samples were dried in a speed vacuum at high temperature for 5 min or until dry. 2.5 µl of formamide loading dye (supplied with the kit) were added to each sample and left for 30 min to allow the DNA to resuspend before running. At this point, samples were kept in the fridge and were ready to be loaded onto the polyacrylamide sequencing gel.

Electrophoresis and fragment detection were carried on an ABI Prism 377 DNA sequencer following the manufacturer's instructions (GeneScan Analysis software Users Manual, The PE Corporation, USA). A 0.2 mm thick 5% denaturing LongRanger (Biowhittaker Molecular Applications, ME, USA) gel was prepared in 1× TBE running buffer (see Appendix I). First, the glass plates (36 cm well to read distance) were washed thoroughly and rinsed with distilled water. Plates were then air dried completely assembled in gel casting cassette before pouring the gel solution. To prepare 50 ml of gel mix, 18 g urea, 26 ml molecular grade water, 5 ml of 50% Long-Ranger solution and 0.5 g mixed bed resin were mixed. The gel mixture was stirred for 30 min to completely dissolve urea and to deionised the solution and this was then filtered through a 0.2 µm Whatman® filter. During filtration, 5 ml of 10× TBE buffer was also added. The filtered gel mixture was degassed by vacuum for 4 min. Then 250 µl of freshly prepared 10% ammonium persulphate (APS in water; Amresco, USA) and 25 µl of N,N,N',N'-Tetramethylenediamine (TEMED; Sigma, UK) was added to the gel solution, mixing very carefully to avoid air bubbles. Using a 50 ml syringe, the gel mix was injected between the glass plates, a comb spacer placed at the upper end and left for a minimum of 2.5 hours to allow polymerisation.

Before the assembling of the glass plates on the ABI Prism 377 DNA sequencer machine, any excess of polyacrylamide gel on the edges was removed with tissue. A shark-tooth comb with 48 wells was inserted at the top of the gel and the assembly was mounted together with the top and bottom buffer chambers, filled with 1,300 ml of 1× TBE buffer. Once the heating block was placed on the glass plates, a pre-run was performed for 45 min or until the

plates reached 45°-50°C. Leaving the machine on pause at this point, 2 µl per sample were loaded and run at 2,500 V, with filter set “F”, for 4 h.

After electrophoresis, the gel image was tracked and the sequences extracted from chromatogram files. The Chromas analysis program v. 1.15 (http://www.technelysium.com.au/chromas.html) was used for manual editing of the DNA sequences and identified vector sequence was trimmed. Sequences were saved in FASTA format and further analysed through BioEdit software v. 7.0 (Hall, 1999), DNAStar software v. 5.0 for windows (Lasergene) and other NCBI based bioinformatics tools.

2.5.2 Sequencing on CEQ 8800 and data analysis

During the project sequencing equipment was upgraded. For later sequencing of PCR products a capillary sequencer CEQ™ 8800 (Beckman Coulter®, USA) and software CEQ™ 8800 v. 9.0 was used, following the manufacturer’s standard protocols for operation and collection of sequence data. GenomeLab™ Dye Terminator Cycle Sequencing with Quick Start kit (Beckman Coulter®, USA) was used, following the recommended procedure for sample preparation and cycle sequencing.

Firstly, PCR products were loaded on small electrophoresis low-melting agarose gels and run under standard conditions (section 2.3.3) to isolate target fragments (see section 2.4.1), which were purified using the Ultra PCR clean-up kit (ABgene™, UK) following the manufacturer’s instructions. After resuspension in water, samples were quantified and concentrations adjusted to

10 ng/µl. At this point, samples were mixed with the Quick Start Master Mix and appropriate sequencing primer to run DNA sequencing reactions (Table 2.10).

Reactions were prepared in 200 µl microcentrifuge tubes. Using a thermocycler, cycle sequencing programme parameters were implemented as described in Table 2.11.

Table 2.10 Components used for sequencing of previously amplified and purified PCR fragments.

Components	Stock Solutions	PCR volumes (µl)
Quick Start Master Mix	1×	2.0
Forward or Reverse primer	5 pmol/µl	1.0
Purified PCR fragments	10 ng/µl	2.0
TOTAL	---	**5.0**

Table 2.11 Cycle sequencing programme parameters for PCR products before final sequencing.

Steps	Cycle Parameters		
Denaturation Annealing Extension	30 cycles	96°C 58°C 60°C	20 seconds 20 seconds 4 seconds
Hold	---	10°C	---

After cycle sequencing, 20 µl of Stop Solution (2 µl of 3 M sodium acetate, 1 µl Glycogen (20 µg/ µl; Roche, Germany), 2 µl 100mM EDTA, pH 8.0 and 15

µl of water) was added and the DNA precipitated by addition of 60 µl of 95% ethanol. The mixture was chilled on ice for 2-3 min. The supernatant was drained and the pellets washed with cold 70% ethanol. The pellet was then dried for 15 min in dark at RT and finally resuspended in 30 µl proprietary Sample Loading Solution (SLS, Beckman Coulter®, USA). The sample was then transferred into a 96 well plate with V bottom (Beckman Coulter®, USA). One drop of mineral oil was added to each well to prevent oxidation of SLS during the sequencing process. An electrophoresis buffer tray, 96 well plate with flat bottom (Beckman Coulter®, USA), was also prepared. Each row of 8 samples ran for 1 hour 20 minutes using the Beckman LFR-1 sequencing method.

Chromatogram files were generated automatically by the software program and sequence analysis was carried out with the same bioinformatics softwares mentioned in section 2.5.1.

2.6 DNA Genotyping

2.6.1 Performance of singleplex PCR reactions

Singleplex PCR reactions were performed for all the markers used for genotyping, following a fluorescent labelled tailed primer method described by Raposo (2001). This was employed as a cost-efficient method. It was not necessary to purchase different fluorescent tagged primers for each locus under investigation.

For each reaction, three different primers were required:

1. An M13 tailed primer (5'Dye-CACGACGTTGTAAAACGAC-3') labelled with one of three CEQ 8800 detectable fluorescent tags (D2-blue, D3-green, D4-blue).

2. Locus specific 'forward' primer with 5' M13 sequence tail.

3. Locus specific 'reverse' primer.

The labelled primers (Proligo, France) were resuspended in 1× TE, pH 8.0 at 100 pmol/µl (stock) concentration.

The PCR master mix and annealing temperatures were optimised for each set of primers, but the standard conditions advised for this protocol are enlisted in Table 2.12 and Table 2.13. The tailed primer:labelled primer molar ratio was in all cases less than or equal to 0.75:1 during optimisation steps.

Table 2.12 PCR formula used for fluorescent labelling of specific fragments (markers) amplified from the genomic DNA by the tailed primer method.

Components	Stock Solutions	Per Reaction	PCR volumes (µl)
Water	---	---	10.00
Buffer IV	10×	1×	1.50
$MgCl_2$	25 mM	1.5 mM*	0.90
dNTPs	5 mM each	0.2 mM each	0.60
Labelled M13	10 µM	4 pmol	0.40
Tailed Primer F or R	5 µM	0.2 pmol*	0.04
Primer F or R	10 µM	4 pmol	0.40
Taq Polymerase	5 U/µl	0.05 U/µl	0.16
Genomic DNA	50 ng/µl	50 ng	1.00
TOTAL	---	---	**15.00**

*** During optimisation conditions, $MgCl_2$ (between 1.5-2.0 mM) and Tailed Primer (between 0.2-2.0 pmol/µl), were the two main factors modified in the master mix composition.**

Table 2.13 PCR programme parameters applied for fluorescent labelling and amplification of specific fragments (markers) by the tailed primer method.

Steps	Cycle Parameters		
Denaturation Annealing Extension	25 cycles	95°C 57°C* 72°C	1 minute
Extension	---	72°C	30 minutes
Hold	---	10°C	---

*** During optimisation conditions, annealing temperature ranged between 57°-60°C and was the only factor modified in the cycle parameters.**

2.6.2 Genotyping

The labelled PCR fragments were genotyped using the CEQ 8800 capillary sequencer. In most cases up to four loci were sequenced simultaneously in multiplex genotyping runs. Generally, PCR products labelled with D4-blue were diluted in water 4 times, D3-green 2 times when combined with D2-black dye products.

For each capillary run, 0.5-0.8 µl of combined or single PCR reactions were added into a 96 well sequence plate with V bottom (Beckman Coulter®, USA) to 30 µl SLS and 0.25 µl DNA Size Standard kit-400 (SS400, Beckman Coulter®, USA) containing fragments labelled with D1-red dye. One drop of mineral oil was added at the top of each sample. An electrophoresis buffer tray, 96 well plate with flat bottom (Beckman Coulter®, USA), was prepared. Each row of 8 samples ran for 45 min using Beckman Frag-3 genotyping method.

2.6.3 Genotyping data analysis

Trace files were generated automatically, and analysed later within the same Beckman-Coulter software (Fragment analysis module) which identified and quantified the detected allelic fragment. All genotype results were transferred to Excel spreadsheets where data was used for further linkage mapping analysis. The Linkage Analysis Package for Outcrossed Families with Male or Female Exchange of the Mapping Parent (LINKMFEX) v. 2.3 software was used for the construction of linkage maps, using the Theta function (no double crossover).

2.7 Chromosome Preparation

2.7.1 Mitotic cell chromosomes

Mitotic cells (white cells or lymphocytes) were obtained from blood of phenotypic adult males and females from the eight fish species mentioned in section 2.2.1, fish stock collection held at the Institute of aquaculture, University of Stirling. Once the fish were anaesthetised with 0.01% benzocaine, blood from the main peduncle arterial was collected with a 3 ml heparin treated syringe (containing about 100 µl of 4 mg/ml heparin stock solution, Sigma, UK), then 1 ml was centrifuged in a microcentrifuge tube at 100 g for 3 min. Following manufacturer recommendations (Invitrogen, cell culture of peripheral red blood cells protocol) with modified optimisation steps, the supernatant was transferred into a 10 ml sterile tube containing 8 ml of PB-MAX™ karyotyping medium (GIBCO™ cell culture, Invitrogen, UK) aiming to transfer also the white layer (lymphocytes) at the top of the concentrated red blood cells. Cell cultures were incubated at 28°C for 3 days, leaving the cap loose and inverting gently once a day.

Working with up to six sample tubes at a time, at the end of the incubation period, 100 µl of Colcemid (10 µg/ml stock solution, Ciba-Geigy, Switzerland) were added and incubated for a further hour at 28°C. For every tube, the cell suspension was spun down at 100 g and most of the supernatant discarded. Leaving about 50 µl of medium, cells were resuspended by gentle stirring, then 8 ml of 75 mM of potassium chloride were added at RT for an incubation time no longer than 30 min as a critical step (*O. niloticus*, 30 min; the other seven species, 20 min). 1 ml of fixative solution (Methanol:Glacial Acetic Acid 3:1) was

added at the end of the incubation time, the tube was inverted once and spun down at 100 g for 5 min at -4°C. The supernatant was discarded. The cells were slowly resuspended in 8 ml of fixative solution, drop by drop, stirring the tube very slowly and then left on ice for 20 min. After centrifugation at 100 g for 5 min at -4°C, the fixation procedure was repeated two more times. As the fixative goes off in about 30 min, fresh fixative solution was prepared each time.

Finally, the cells were resuspended in a small volume (50-100 µl depending on cell yield) of fixative solution, aiming to produce a cloudy solution, and the quality of the cells was checked by dropping 8 µl of the solution onto a glass slide, waiting until dry and observing under a phase contrast microscope at 40× magnification. If successful, chromosome groups were observed with well separated cells and free of cytoplasm, and other blood cells in other stages of development.

2.7.2 Meiotic cell chromosomes

Meiotic cells were obtained from fragments of testes coming from XY premature (3 to 5 months) or adult males, following the protocols of Foresti *et al.* (1993) and Campos-Ramos (2001) for the preparation of Synaptonemal Complex (SC) spreads with some modifications as described above. Fish were killed by an overdose of anaesthesia (>0.01% benzocaine solution) followed by destruction of the brain before dissection. Testes were removed and placed in Hanks' saline solution (Sigma-Aldrich, UK). Using two razor blades, excess fat and external gonadal membranes were stripped off and the remaining tissue washed in at least three changes of Hanks' saline solution to remove of most of

the sperm cells. Tissue was then minced into small fragments and submerged in fresh Hanks' solution. 200 µl of the resultant cell suspension were removed and transferred to a 1.5 ml microcentrifuge tube to be kept on ice for one hour. The tube was centrifuged at 100 g for 2 min and then the supernatant transferred into a new microcentrifuge tube to be centrifuged at the same speed for 8 min. 20 µl from the bottom of the tube (including the concentrated cells) were carefully removed and transferred into a tube containing 60 µl loose pellet spreading medium (0.2% Triton X®; Sigma-Aldrich, UK, in water adjusted to pH 8.5 with 0.01 M sodium tetraborate) and 40 µl of 0.2 M sucrose solution, gently mixed and incubated at RT for 10 min. After incubation time, cell suspension was fixed with 90 µl of 4% paraformaldehyde (buffered to pH 8.5 with 0.2 M sodium tetraborate) and incubated at RT for 90 min. 90 µl of the fixed cell suspension was spread onto a Superfrost® Plus grass slides (Menzel-Gelaser Limited, Germany); samples were left to dry for about 2 h in a vertical position in a fume cupboard. Finally slides were rinsed with distilled water first and then with 95% ethanol before the last air drying and observation under a phase contrast microscope at 40× magnification to check the quality of the cells, aiming to see well dispersed and abundant chromosome groups free of cytoplasm and a low abundance of sperm and debris.

2.8 Fluorescent In Situ Hybridisation (FISH)

2.8.1 Slide preparation for mitotic cells

Superfrost® Plus glass slides (Menzel-Gelaser Limited, Germany) were pre-cleaned by immersion in 70% ethanol for 1h prior to use. Slides were wiped

dry with a lint-free tissue (Agar Scientific, UK) before dropping 12 µl of cell suspension (mitotic cells) in the centre of the slide, allowing it to spread and air dry.

After about 20 min, slides were dehydrated through an ethanol series (70%, 80%, 95% and 100%) using coplin jars (Thistle Scientific, UK) for 3 min each. The next step was considered to be critical to allow DNA probes to penetrate into the chromosomes but at the same time to try keeping their general morphology, slides were denatured for exactly 70 sec at 70°C in denaturing solution (70% formamide in 2× SSC, see Appendix I). Immediately after the denaturation step, slides were quenched in 70% ethanol at -20°C for 3 minutes. Another ethanol series (3 min each in 80%, 90% and 100%) for dehydration followed.

Finally slides were air dried and at this point they were ready for a subsequent addition of probe + blocking DNA.

2.8.2 Slide preparation for meiotic cells

In the case of meiotic cells, spreads were prepared straight away onto the slides to be hybridised. Dehydration and denaturation steps then took place as described in section 2.8.1.

2.8.3 Probe and blocking DNA preparation

BAC construct probes were prepared using the Nick-Translation technique (Kessler, 1992; Rigby and *et al.*, 1977). Nicks are induced by DNAse I at low

concentrations, removing dNTPs using its 5' to 3' exonuclease activity; as it removes a 5' –residue at the site of the nick, it adds a nucleotide to the 3' –end, incorporating Biotin-16-dUTP or Digoxigenin-11-dUTP nucleotides using its polymerising activity. Moreover, in the presence of $MgCl_2$, the process generates a fragment smear of variable sizes.

Details about probe preparation are as follows: 10-12 µl of a 100 ng/µl target BAC construct was mixed with 5 µl of 4× Dig/Bio-Nick Translation Mix (ROCHE, Germany) and adjusted with water for a final volume of 20 µl. The mixture was incubated in at 15°C until resultant fragments were in 200-700 bp size range. This normally took 20-25 min. After 20 min the reaction was put on ice and 3 µl aliquot electrophoresed on 1.5% agarose gel. If necessary the reaction was continued at 15°C for a further 5 min when required. The enzymatic reaction was stopped with 1 µl of 0.5 M EDTA and heated at 65°C for 10 min.

DOP-PCR (Telenius *et al.,* 1992) was used to randomly amplifying tilapia genomic DNA (for blocking DNA) under specific PCR conditions. A DOP universal primer with the following sequence: 5'-CCGACTCGAG $(N)_6$ ATGTGG-3' was employed.

Details of DOP-PCR preparation are given in Table 2.14 and Table 2.15. Multiple PCR reactions were undertaken to produce sufficient blocking DNA for all experiments.

Table 2.14 PCR formula applied for blocking DNA preparation by DOP-PCR.

Components	Stock Solutions	Per Reaction	PCR volumes (µl)
Water	---	---	9.35
Buffer IV	10×	1×	1.50
$MgCl_2$	25 mM	1.5 mM	0.90
dNTPs	5 mM each	0.2mM each	0.60
DOP Primer	10 µM	1 µM	1.50
Taq Polymerase	5 U/µl	0.05 U/µl	0.15
Genomic DNA	300 ng/µl	300 ng	1.00
TOTAL	---	---	**15.00**

Table 2.15 PCR programme parameters applied during blocking DNA preparation.

Steps	Cycle Parameters		
Denaturation Annealing Extension	30 cycles	94°C 60°C 72°C	30 seconds 100 seconds 45 seconds
Hold	---	4°C	---

Both final probe and blocking DNA were combined as 10 µl of probe solution (50 ng/µl stock solution) and an excess of degenerate tilapia genomic DNA (50 µl of DOP-PCR Blocking DNA). The 60 µl mixture was concentrated in a Speedvac® DNA 110 (SAVANT Technologies) at medium temperature for 20 min until nearly dry and resuspended in 20 µl hybridisation solution for *in situ* hybridisation (containing dextran sulfate and formamide; Sigma-Aldrich, UK). When dual hybridisation was required (using two different probes at the same time), 10 µl of each probe was added together with a double amount of blocking DNA, making 120 µl mixture; the evaporation step ran for 45 min and

resuspension was still in 20 µl Hybridisation mixture. The final concentration of each probe was about 25 ng/µl.

The probe mixture incubated at 42°C for one hour with occasional mixing (at least twice), then denatured at 75°C for 5 min and held at 37°C for 15 min to allow self binding of non-specific fragments. The sample was then ready to be added carefully onto the slide either with mitotic or meiotic preparation. The slide was covered with LifterSlips™ premium cover slip (Erie Scientific Company, USA), avoiding bubbles formation, and placed into a sealed moist chamber (Genetix, UK) at 37°C overnight.

2.8.4 Antibody detection

Three coplin jars were prepared with 50% formamide in 2× SSC and two coplin jars with 2× SSC and allowed to equilibrate in a water bath at 42°C. Removing the hybridised slide from the moist chamber, it was placed into the first coplin jar with 50% formamide solution for 5 min to allow the cover slip to detach, and then the slide was rinsed in the second and third jar consecutively for another 5 min each.

Next, the slide was washed in two changes of 2× SSC solution at 42°C for 5 min each and then placed into 4× SSC containing 0.05% (v/v) Tween®20 (Detergent, Sigma-Aldrich, UK) and left for another 15 min at RT. After this time, the slide was treated with 200 µl of 3% BSA in 4× SSC and 0.05% (v/v) Tween®20, blocking non-specific antibody binding, and covered with a clean cover slip before incubation at RT for 30 min.

Biotin/streptavidin (FluoroLink™Cy3 Labelled streptavidin; Amersham Biosciences, England) and digoxigenin/anti-digoxigenin antibodies (anti-DIG-FITC) (Anti-Digoxigenin-Fluorescein Fab Fragments; ROCHE, Germany) were used for the detection of fluorescence signal. The detection solution was 1 µg/ml streptavidin and 1 µg/ml anti-digoxigenin in 4× SSC, 0.05% Tween®20 and 1% BSA. Two hundred µl were prepared at a time, and then the mixture centrifuged at 10,000 g for 5 min. Finally, 180 µl were taken from the mixture and the slide covered again with a clean cover slip and incubated in a moist chamber at 37°C for 40 min.

The slide was rinsed twice in 4× SSC containing 0.05% Tween®20 at 42°C for 5 minutes, rinsed briefly in distilled water, air dried for 1 h, counterstained with one drop of DAPI-Antifade compound mounting medium (DAPI; 4',6-diamidino-2-phenyl-indole, 0.3 µg/ml) (Vectrashiend®, Vector Laboratories, Burlingame, CA) and a cover slip added just before observation under 100X magnification on a fluorescent microscope fitted with appropriate filters.

2.8.5 Visualisation and analysis

A fluorescence inverted microscope (Olympus 1×70; Olympus, UK) was used for the observation of one or two different probes along with the chromosomes simultaneously, taking about 20 digitalised pictures from different metaphase or SC spreads from each analysed slide.

A drop of immersion oil was applied onto the 100× objective lens and the mounted slide placed inverted on the microscope stage. Images were visualised with a high intensity fluorescent light (100watts mercury arc lamp)

and using DAPI, Cy3 and FITC filters with wavelengths of 360, 568, 488 nm respectively. Fluorescent signals were screened, captured and pseudocoloured using a digital camera attached to the microscope and a digital imaging system (CytoVision 2.7; Applied Imaging International, UK), giving a compiled image with a blue colour background for DAPI, red colour for Cy3 and green for FITC.

True gene marker or gene localisation was defined for signals present on each homologue of a chromosome pair with either two signals on the chromosome (both chromatids) or one signal representing the two chromatids.

2.9 Statistical Analysis

The most common statistical analyses used in the main chapters of this research (Chapters 3, 4, and 5) are listed below.

Analysis of survival rates by one way Analysis of Variance (ANOVA) test was performed to compare the survival at different stages of embryonic development among the different family crosses. SPSS (v. 11 for Windows) statistical analysis software was used for this purpose.

Chi-square analysis was calculated (excel formula) for genotypic determination of parents according to Mair *et al.* (1994), following pooled progeny data (proportion of males:females) for every fish family created.

Finally, Goodness-of-fit was calculated according to Mendelian expectations and linkage maps constructed, using LINKMFEX software v. 2.3 downloaded from R. Danzman's website (http://www.uoguelph.ca/~rdanzman/software/LINKMFEX/) and graphical representations of linkage groups created with MapChart 2.2 (http://www.biometris.wur.nl/UK/Software/MapChart/) (Voorrips, 2002).

Chapter 3 PHYSICAL MAPPING

3.1 Summary

In this chapter the physical distribution of markers and genes associated with sex determination in Nile tilapia *O. niloticus* were analysed. The aim was to construct an accurate physical map based on fluorescent *in situ* hybridisations (FISH) of probes from 23 Bacterial Artificial Chromosome (BAC) clones containing sequences of interest, such as sex-linked AFLPs and microsatellites; insulin, ovarian aromatase and DMO genes; and markers associated with the dominant red skin colour trait.

A comparison of the *O. niloticus* physical map was made with seven other tilapia species (*O. aureus, O. karongae, O. mortimeri, O. mossambicus, Sarotherodon galilaeus, Tilapia rendalli, T. zillii*) using the same set of markers.

With the collaboration of Dr. Konrad Ocalewicz and Dr. Rafael Campos-Ramos, the orientation of chromosome 1 in *O. niloticus* according to the unpaired region, observed in the Synaptonemal Complex (SC) spreads from XY animals, was confirmed as previous authors suggested. The orientation was achieved by a combination of DAPI staining of centromeres and two BAC clones.

Additionally, detailed karyotypes were made based on mitotic spreads from *O.niloticus*, and mitotic and meiotic spreads from *O. karongae*. Moreover, in collaboration with Dr. Cesar Martins, it was possible to discriminate five of the largest chromosomes by identifying fusion points in chromosome 2, 3 and 4 by FISH using repetitive sequences (SATA) and BACs from three selected linkage groups (LG1, 3 and 7). In contrast, hybridisation with telomeric sequences did

not show clear evidence of interstitial (close to the middle of the chromosome arms) signals on these two species.

3.2 Introduction

3.2.1 Cytogenetics and sex chromosomes

More than 1700 species of fish have been cytogenetically characterised and approximately 176 species or 10.4% have been found to have cytogenetically distinct sex chromosomes (Devlin and Nagahama, 2002). A reason for this low proportion (a minimum estimate) are that many of the studies quoted were not looking specifically for sex chromosomes, or due to hermaphroditism, polygenic sex determination system, etc.; and in many other cases, cytogenetic differences between heteromorphic pairs are too small to be detected by conventional cytogenetic techniques (Gold *et al.*, 1980). In this way, SC studies, DAPI staining and FISH are helping to identify these possible differences in heterogametic animals (Belonogova *et al.*, 2006; Carrasco *et al.*, 1999; Harvey *et al.*, 2002b). According to Devlin and Nagahama (2002), more than twice as many species display male heterogamety compared to female heterogamety. They suggested that XY systems of sex determination could favour the fitness of the species positively correlated with male size, whereas ZW systems occur with female size advantages; even though, there are several exceptions to this rule.

Karyotypes are quite dynamic as demonstrated by differences in chromosome number or morphology between closely related species. Changes in chromosome number could be related to Robertsonian translocations, the

most common kind of centric fusion. This is the union of two centromeres commonly from acrocentric chromosomes giving as a result a single metacentric or submetacentric (Winter *et al.*, 2002).

It is broadly accepted that sex chromosomes become differentiated gradually. This process begins with a suppression of recombination during meiosis due to a mutation or mutations that become "fixed" in the population, leading to an eventual functional and structural degradation of the Y (or W?) chromosome (Ayling and Griffin, 2002). Devlin and Nagahama (2002), mentioned that this process does not necessarily follow a one way direction, but is a dynamic process whereby sex chromosomes and other polygenic factors increase and decrease in their influence over the sex determination system, moving the sex control backward and forward between monogenic and polygenic control. Ohno's law and Muller's ratchet describe theories of intermediate processes of chromosomal sex differentiation (Charlesworth, 2002) that elucidate clear intermediate alternatives during the evolution of sex differentiation systems (Charlesworth, 2002) such as in snakes (ZZ/ZW system) and the reindeer *Rangifer tarandus* (XX/XY system) (Ayling and Griffin, 2002). Despite an apparent multidirectional sex differentiation processes, the "threshold dichotomy" theory of Mittwoch (2006) proposes that contrasting phenotypes (males and females) have their origin in multiple genes with quantitative effects and are separated by a physiological threshold. In mammals, the Y chromosome minimises the effect of these variables on XY embryos and with few exceptions the inheritance of sex in mammals still resembles a single gene effect.

The transcriptionally inert chromatin regions in chromosomes are called heterochromatin. Heterochromatin consists of two types (Czepulkowski, 2001); a) Facultative heterochromatin, which can be genetically active or inactive; b) constitutive heterochromatin which is always inactive. These regions contain repetitive sequences similar to the ones described in centromeric regions and in the p arms of acrocentric chromosomes, revealed by C-banding (Winter *et al.*, 2002). Heterochromatinisation is described as the process of accumulating repetitive sequences, and this inhibits recombination between homologous chromosomes. This is one of the main reasons why heterochromatinisation is very often related to sex chromosome differentiation as demonstrated in more evolved terrestrial animals (Ayling and Griffin, 2002) as well as in early stages of sex differentiation in aquatic animals (De Almeida-Toledo *et al.*, 2001b).

In Nile tilapia, a genetic sex determination system has been identified with an XX female and XY male (Mair *et al.*, 1991a). There is evidence for partial penetrance with feminisation or masculinisation effects from autosomes in some cases (Sarder *et al.*, 1999) and masculinisation effects of high environmental temperatures in others (Abucay *et al.*, 1999; Baroiller *et al.*, 1996). Analysis of meiotic chromosomes of *O. niloticus* revealed an unpaired region on the largest bivalent, only present in XY animals but not in XX or YY, indicating that recombination is inhibited between X and Y chromosomes (Carrasco *et al.,* 1999; Griffin *et al.,* 2002), a phenomenon that is required for the evolution of differentiated sex chromosomes (Lorch, 2005; Rice, 1987). Cross hybridisation of probes made from the distal part of the long arm (by microdissection of chromosome 1) from X and Y chromosomes demonstrated sequence differences (Harvey *et al.,* 2002b) although the available evidence

suggests that only limited genetic differences exist between the X and Y chromosomes (Ezaz *et al.*, 2004b). The demonstration of a high heterochromatinisation area in the distal second part of the q arm on chromosome 1, as revealed by satellite DNA CiLINE2 hybridisation (Oliveira *et al.*, 1999), and the physical mapping of sex-linked Amplified Fragment Length Polymorphism (AFLP) markers around the middle of the same arm (Ezaz *et al.*, 2004b), has reinforced the hypothesis that a sex determination area resides on chromosome 1. Because there is no evidence of morphological differences between this pair of homologous chromosomes, it was suggested that sex chromosomes in tilapia are in early stages of differentiation (Harvey *et al.*, 2002b).

3.2.2 Origin of the markers and genes used

There are four main types of probes commonly used during FISH studies (Czepulkowski, 2001); unique sequence probes, alpha satellite probes, telomeric probes and whole chromosome paints. In this chapter unique sequence probes and alpha satellite probes were employed.

Unique sequence probes range from very small single copy probes to large inserts such as the BACs which can accommodate foreign DNA fragments typically between 50 to 500 kb (Tamarin, 2002). These sequences are usually locus-specific and can detect a specific chromosomal region. Amplified Fragment Length Polymorphism (AFLP) markers, microsatellite markers and genes can be identified from within Phage BAC insert commonly

by dot-blot or southern-blot hybridisation of subcloned fragments and these used to localise the marker or gene onto mitotic or meiotic chromosome plates.

Alpha satellite sequences are made up of tandem repetitive DNA mainly found in the satellite centromeric region of all chromosomes. They are also termed satellite DNA (SATA). Telomeric sequences are found at the end of the telomeres from each chromatid, known as telomeric sequences $(TTAGGG)_n$. Alpha satellite probes and telomeric probes are also characterised by generation of a very bright signal on FISH. However, the stringency of the post-hybridisation washes has to be very well controlled to avoid cross-hybridisation artefacts. The importance of SATA sequences lie on their strong association with heterochromatin accumulation along the genome (Winter *et al.*, 2002), and telomere sequences because of its conserved tandem repeats among many different animal species (Molina and Galetti, 2002; Silva and Yonenaga-Yassuda, 1998; Sola *et al.*, 2000). There are also other type of satellite probes such as ribosomal DNA genes (rDNA), Short Interspersed Elements (SINEs) and Long Interspersed Elements (LINEs), found distributed interstitially on specific chromosome arms or almost homogeneously along all the chromosomes. These satellite probes are particularly useful for identifying marker's specific chromosomes in metaphase preparations (Martins *et al.*, 2004; Phillips, 2001).

A review on the repetitive DNA isolated from cichlids has been made by Phillips and Reed (1996) and Martins *et al.* (2004) localising not only SATA probes but also telomere, 45S and 5S rDNA, and the short and long interspersed nucleotide element (SINE and LINE respectively) probes to

centromeres, telomeres and putative sex chromosomes. Specially, centromeric and telomeric probes have been used to detect intraspecific chromosome rearrangements in other fish species such as salmonids (Phillips, 2001). Some of the cichlid species initially analysed were *Oreochromis niloticus* (Franck *et al.,* 1991)*, O. hornorum* (Franck and Wright, 1993)*, Tilapia rendalli* and *T. zillii* (Franck *et al.,* 1992)*,* and *Sarotherodon galilaeus* (Wright, 1989).

In tilapia, some specific satellite sequences had been revealed like SATB (1900bp monomer size) and CiLINE2 (1165bp monomer size), the former being found on the short arm of chromosome 4 and the later enriched in the long arm of chromosome 1 (Franck and Wright, 1993; Oliveira *et al.,* 1999). In *O. niloticus*, SATA (237bp monomer size) and telomeric sequences have also been identified (Chew *et al.,* 2002; Oliveira and Wright, 1998), SATA gave interstitial signals in the short arms of two chromosomes and telomere probes two interstitial bands on the long arm of chromosome 1, suggesting the fusion of three small chromosomes to create the largest chromosome of the karyotype.

Returning to the subject of the unique sequence probes applied in this research, it is widely accepted that during the construction of genomic maps, microsatellites and AFLPs are the preferred markers of choice due to their high level of polymorphism. These molecular markers have been used in different species to identify sex-specific markers or sex-linked markers. The molecular markers closely linked to the sex locus have allowed reliable and quick identification of sex at early stages of development such as the Xmrk locus in *Xiphophorus maculatus* (Coughlan *et al.,* 1999), DMY in *Oryzias latipes*

(Matsuda *et al.,* 2002) and two Y-chromosome linked AFLP markers in the threespined stickleback (Griffiths *et al.,* 2000). However, the differences between sexes in fish are often limited and not conserved within closely related species (Devlin and Nagahama, 2002). Three AFLPs described as Sex-Linked AFLP Markers (SLAMS) (Ezaz *et al.,* 2004b) were developed specifically for Nile tilapia based on the differences found between X and Y chromosomes, from homozygous XX and YY families produced by gynogenesis from XY neofemales. The SLAMS developed proved to be sex-linked in normal crosses by 13 cM in the case of *OniY*382, 17 cM for *OniY*227 and 20cM for the allelic marker *OniX*420/*OniY*425. They represent good candidates to serve in a physical map as references to find the sex locus.

Using bulked segregant analysis, ten microsatellites belonging to LG1 were found to be linked to phenotypic sex in *O. niloticus* (XY system), predicting 95% of males and females in two families, but none in a third one, suggesting that additional genetic and environmental factors regulate sex determination in this species (Lee *et al.,* 2003). Microsatellites UNH995 and UNH104 were determined to be one or less centimorgans apart from the sex locus. Using bulked segregant analysis in *O. aureus* (WZ system; Lee *et al.*, 2004), 11 microsatellites were found from LG3 that correctly predicted progeny sex (97% of males and 85% of females), suggesting a putative W chromosome within a few centimorgans of the markers GM354, UNH168, GM271 and UNH131. Interestingly, markers from LG1 also showed some association with sex leading to a proposal of an epistatic interaction of loci between the dominant male repressor (on LG3) and a dominant male determiner (on LG1).

3.2.3 Development of physical maps in tilapia

Physical maps are one of the three kinds of genome maps constructed for species, localising segments onto the karyotype by cytogenetic methodologies. The second are genetic linkage maps that assign the linear order of DNA markers along a chromosome using recombination frequencies between loci. The third are sequences of a genome and its integration with physical and genetic maps that provides the final complete genetic map.

The efforts for this chapter were focused on the development and integration of a physical map in tilapia for a further comparison with genetic maps and further localisation of Quantitative Trait Loci (QTL) with relevance in aquaculture. Mapping also opens up the possibility of comparative mapping among fish species and facilitates the assembly of genomic shotgun sequences. As mentioned by Martins *et al.* (2004), this represents the best strategy to get a better understanding of the structure and evolution of the cichlid genome.

Fluorescent *In Situ* Hybridisation (FISH) has been used to map highly repetitive sequences including sequences specific for centromeres, sex-specific and sex-linked sequences, moderately repetitive sequences (rRNAs and histones), and single-copy sequences as described in the previous section. This kind of physical map has been developed in fish models such as zebrafish (Phillips and Reed, 2000), pufferfish (Fischer *et al.,* 2000), medaka (*Orizias latipes;* Matsuda *et al.,* 1998), platyfish (*Xiphophorus maculata*; Nanda *et al.,* 2000), tilapia (*Oreochromis niloticus*; Oliveira *et al.,*1999), rainbow trout (*Oncorhyncus mykiss*; Reed *et al.,* 1998), and other salmonids (Phillips, 2001).

Studies on F2 generations of species have combined physical and genetic map data such as rainbow trout (Phillips *et al.,* 2006b) and zebrafish (Phillips *et al.,* 2006a).

To assist in the construction of a physical map in Stirling Nile tilapia, 4 BAC libraries with different mean insert sizes (TBL1 with 65kb, TBL2 with 105kb, TBL3 with 145kb and TBL4 with 194kb) have been constructed and characterised (Katagiri *et al.,* 2001). These BACs libraries are also known as HCGS-01TI, USTI-02TI, HCGS-03TI and HCGS-04TI respectively (Hubbard Centre for Genomic Studies, University of New Hampshire). The estimated coverage is 6x, 65x, 11x and 6x respectively for the four tilapia BAC libraries.

These libraries opened the possibility of constructing a detailed physical map in tilapia. A genome-wide physical map (BAC-based, linking collections of BAC clones by fingerprinting) was constructed from the tilapia genome based on restriction fingerprints of more than 35,000 large insert BAC clones. This map consists of 3,621 contigs, estimating to span 1.752 Gigabases (Gb) in physical length (Katagiri *et al.,* 2005). This detailed map is a valuable resource for both positional cloning and assembly of the whole genome sequence.

To date, a physical map has been developed using several repetitive DNA sequences (SATA, SATB, telomere sequences, CiLINE2, Ron-1 and 2, 45S rDNA, 5S rDNA) in Nile tilapia (Martins *et al.,* 2004). Specific DNA sequences such as copies of DMO (DM DNA binding domain named after the two genes, *doublesex, dsx* and *male-abnormal 3*, *mab-3*, both recornised in proteins encoding sex-determining genes; Shen and Hodgkin, 1988; Burtis and Baker, 1989), SOX (named after the SRY- related HMG box transcription factor gene

family containing a conserved region related to the DNA binding domain of the mammalian testis determining gene Sry; Sinclair *et al.*, 1990) and Insulin genes together with three SLAMs mentioned before have been mapped on chromosome 1 (Boonphakdee, 2005; Ezaz *et al.,* 2004b). Very recently, several microsatellites and genes were also mapped on LG3 and LG1 according to Lee's linkage map (Lee *et al.,* 2005), e.g. UNH995/UNH104 (contained into the same BAC clone) mapping to a small chromosome; GM354, UNH168, clcn5, GM150, trp1, GM128 and others mapped on chromosome 1. All these markers and genes have been demonstrated to be sex linked in tilapia species (Cnaani *et al.,* In review). Finally, CyP19A1 ovarian aromatase and brain aromatase genes have been mapped by FISH (Harvey *et al.,* 2003a) onto chromosome 2 and a small chromosome pair respectively. All these markers were physically mapped exclusively on Nile tilapia, with the exception of some DMRTs and SOX that were also mapped in *O. mossambicus* and *O. aureus* (Boonphakdee, 2005).

Several of these markers and genes associated with sex and apparently located on different genome regions have been included in this project (mainly from LG1 and LG3). They were physically mapped by dual hybridisation to generate accurate LG assignments and integrate them to the existing cytological physical map in Nile tilapia. Mitotic and meiotic physical maps were also compared with other tilapia species.

3.2.4 Species selected for mitotic analysis

From 70 species of cichlids composing the named group “tilapia”, only *O. niloticus*, *O. mossambicus* and *O. aureus* and their hybrids have been widely studied and cultured around the world. *O. niloticus* is the most studied and despite the advances in genetic studies, knowledge is still considered preliminary in comparison with pufferfish and zebrafish species (Martins *et al.,* 2004).

Previous cytogenetic studies in tilapia species have shown that the haploid genome of *O. niloticus* consists of 22 chromosomes (2n=44) (Kornfield *et al.,* 1979; Majumdar, 1986) and with an XY sex chromosome system (Jalabert *et al.,* 1974). Other Cichlid species such as *O. mortimeri, O. mossambicus, S. galilaeus and T. zillii* (Majumdar, 1986) showed the same number of chromosomes and an XY genetic sex determination system observed. *T. rendalli* also presents 2n=44 chromosomes but with a sex chromosome system still not determined (Foresti *et al.,* 1983). *O. aureus* has a WZ sex determining system (Campos-Ramos *et al.,* 2001) but still presenting 2n=44 chromosomes (Avtalion and Don, 1990). Despite this tilapia group having a general highly conserved karyotype (Majumdar, 1986), differences have been observed in the number of chromosomes such as *O. karongae* with 2n=38 (Harvey *et al.,* 2002a) and with a WZ sex determining system (Cnaani *et al.,* In review).

Having a combination of the two most common genetic sex determining systems, XY and WZ, and different number of total chromosomes 2n=44 and 2n= 38, and the representation of three relative closely related tilapia genera, *Oreochromis, Sarotherodon* and *Tilapia*, the previous species mentioned above

represent ideal model species to study the differences in marker and gene distribution and to understand the proposed different levels of speciation towards sex determination systems.

3.2.5 Species selected for meiotic analysis

Due to different chromosome number, *O. niloticus* (2n=44) and *O. karongae* (2n=38) were chosen to extend a comparative analysis based on the assignment of linkage groups to their chromosomes and to develop karyotypes with better resolution based on SC spreads.

Existing karyotypes from *O. niloticus* (Majumdar, 1986) and *O. karongae* (Harvey *et al.,* 2002a) from mitotic metaphase preparations reveal very close similarity among the small chromosomes. It is only possible to reliably identify chromosomes 1 and 2 in *O. niloticus* and chromosomes 1 to 5 in O*. karongae*. The rest of the chromosomes are very similar in size and shape. It should be possible to improve chromosome identification in the species by developing meiotic prophase (pachytene) karyotypes based on FISH techniques. A similar approach has been successful for other organisms (Cheng *et al.,* 2001) (Peterson *et al.,* 1999). This approach not only facilitates the assignment of linkage groups to a particular chromosome but also helps to orientate them even with a set of tightly linked markers.

Phenomena such as Robertsonian fusion and two other fusions proposed in *O. karongae* (Harvey *et al.,* 2002a) widen the interest on this species to identify by FISH any remnant signal from the chromosome ancestors, and compare them with the closely related species *O. niloticus*.

3.2.6 Objectives

With the purpose to gain a better understanding on the distribution and association of repetitive DNA, markers and genes involved in the sex determination system, the objectives of the present study were as follows:

- To develop an accurate physical map along the putative sex chromosome (chromosome 1) in tilapia based on new and existing genetic markers and genes associated with this chromosome.
- To assign onto chromosomes other putative sex-linked or related markers and genes not present on chromosome 1.
- To compare *O. niloticus* linkage group assignments against seven other tilapia species.
- To develop more accurate karyotypes for *O. niloticus* and *O. karongae* species.

3.3 Materials and Methods

The experimental design is graphically described below (Figure 3.1 and 3.2). It has two main themes: Mitotic studies and meiotic studies. The first theme had the purpose of developing physical maps for seven different species with 2n=44 and integrating them into a single and robust physical map. These maps were based on data from FISH on mitotic chromosomes using BAC clones containing Sex-Linked AFLPs, microsatellites, genes, repetitive DNA (SATA) and telomeric DNA markers. In addition, an interesting comparison was made with *O. karongae* (chromosome number 2n=38) by physically mapping a

few markers, representative of different linkage groups, and repetitive DNA (SATA), trying to identify more accurately the first five larger chromosomes and the type of fusions they represent.

The second theme aimed to study two karyotypes in more detail (*O. niloticus* and *O. karongae*) using a novel way to characterise chromosomes more accurately. Spermatocyte meiotic chromosomes at pachytene stage (SC observations) were analysed with DAPI staining and FISH techniques. The orientation of the unpaired region on the larger bivalent from an XY male Nile tilapia was confirmed and measured during this study.

SATA and telomeric DNA probes were hybridised (in collaboration) by Dr. Cesar Martins (Instituto de Biociencias, Universidade Estatal Paulista, Brazil); the preparation of meiotic chromosomes along with some of the *in situ* hybridisations were done with the collaboration of Dr. Konrad Ocalewicz (Faculty of Environmental Sciences and Fisheries, University of Warmia and Mazury in Olsztyn, Poland) and Dr. Rafael Campos-Ramos (Centro de Investigaciones Biologicas del Noreste, La Paz, Mexico).

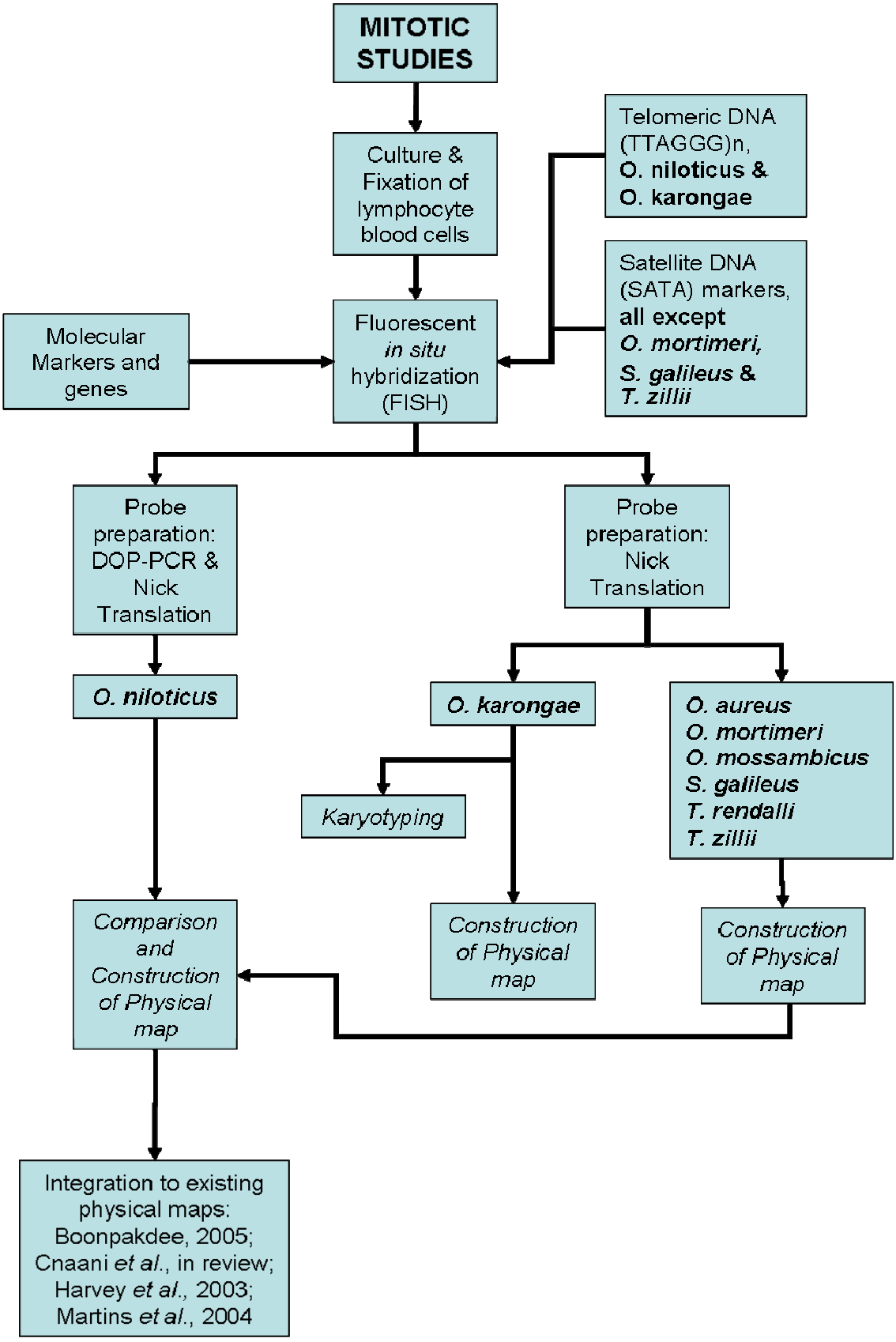

Figure 3.1 Flow diagram representing the steps covered using mitotic studies. It shows how FISH analyses from Nile tilapia *O. niloticus* were compared with seven other species.

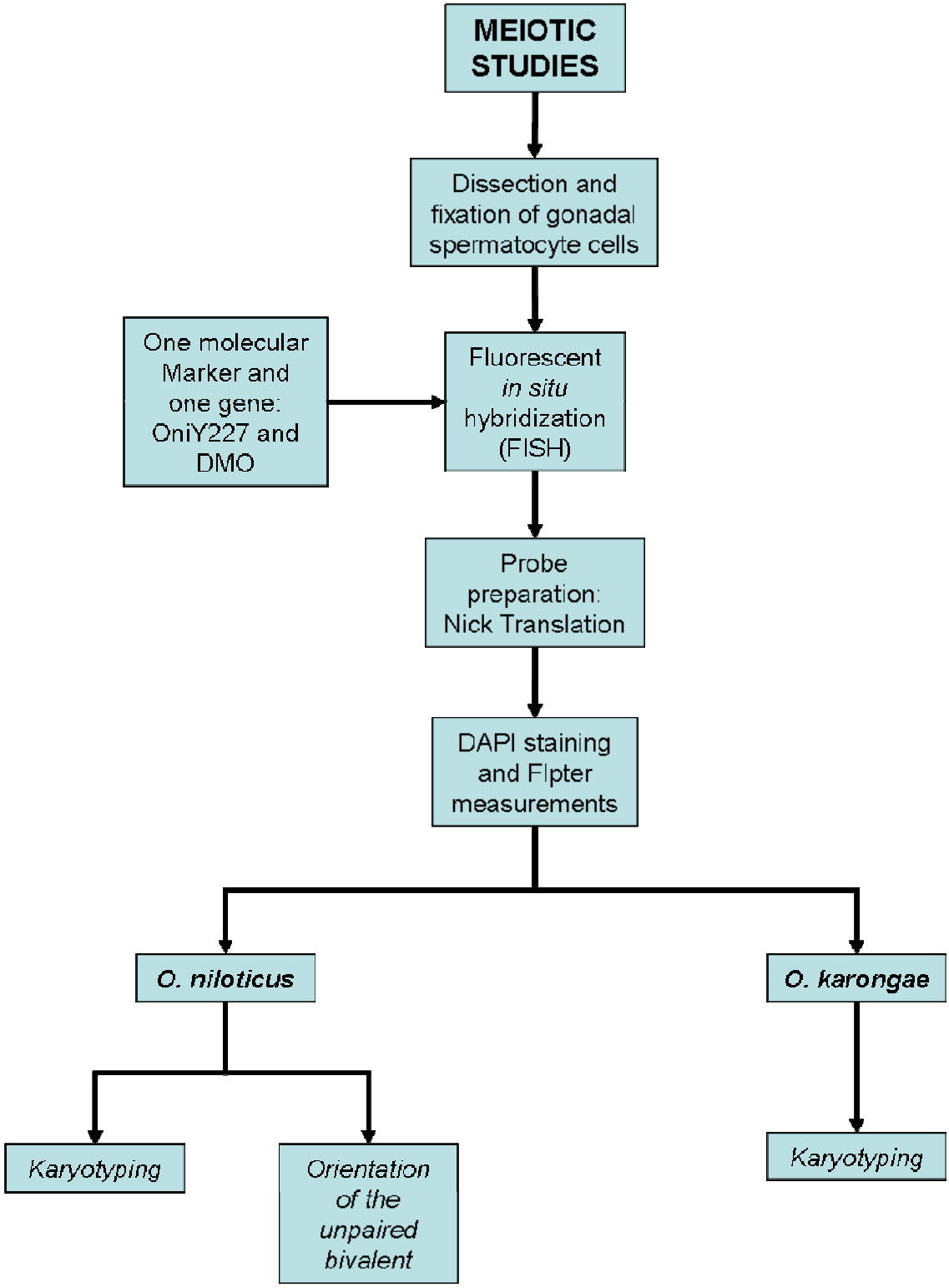

Figure 3.2 Flow diagram representing the steps covered by meiotic studies. One molecular marker and one gene from chromosome 1 were taken for physical mapping for orientation of the unpaired bivalent (chromosome 1). Finally, based on a combination of FISH and DAPI staining for molecular markers and centromere FIpter measurements, two accurate karyotypes were developed from *O. niloticus* and *O. karongae*.

3.3.1 Fish stock used and chromosome preparation

The fish stocks used for mitotic and meiotic studies were held in the tropical aquarium at the Institute of Aquaculture, University of Stirling. Six ordinary males from *O. niloticus*, four males and four females from *O. aureus, O. karongae, O. mortimeri, O. mossambicus, S. galilaeus, T. rendalli and T. zillii,* were selected for blood sampling and lymphocyte culture for mitotic studies (phenotypic sex was confirmed in all the cases by gonad dissection). Five ordinary males from *O. niloticus* and *O. karongae* were chosen for gonad dissection for meiotic studies using, in most cases, the same fish as used for blood sampling (see section 2.7 for a description of mitotic and meiotic cell preparation).

3.3.2 Selection of BAC clones for FISH

BAC clones used in the construction of the tilapia physical map came from three tilapia BAC libraries, two held in the University of New Hampshire, Hubbard Centre for Genomic Studies (Library ID: HCGS-03TI and HCGS-04T1) and one held in the University of Stirling, Institute of Aquaculture (Library ID: USTI-02TI). The three BAC libraries were constructed from the sperm of a single male (no.00-0135-EA1B) from a strain originating from Lake Manzallah, Egypt and maintained at the University of Stirling, UK (Katagiri *et al.,* 2005).The mean insert sizes for the three BAC libraries are 145Kb, 194Kb and 105kb respectively.

One set of 32 BAC clones containing 14 microsatellites and 7 genes was selected from the BAC libraries of Nile tilapia HCGS-03T1 and HCGS-04T1

according to their linkage groups and clear or potential relation with the sex determination system. Previous linkage maps developed by Kocher *et al.* (1998) and Lee *et al.* (2003; 2004; 2005) showed that linkage groups included in this study were LG1, LG3, LG6, LG7, LG10 and LG12. Appendix V shows complete references from each of the microsatellites and genes used, including information about the different projects and contigs related with these 32 BAC clones.

A second set of markers and genes physically mapped in this project, linked or associated with the sex determination system, were isolated and identified previously by Harvey *et al.* (2003b), Ezaz *et al.* (2004b) and Boonphakdee (2005) from BAC clones belonging to the BAC library USTI-02TI. In this case, most of the BAC clones included from this library were previously mapped onto chromosome 1 and only one onto chromosome 2. The set of BACs included three Sex-Linked AFLP Markers (SLAMs), insulin, DMO (dmrt4), DMT (dmrt1,2,3), Sox9b and ovarian and brain aromatase genes. Appendix VII shows complete references for each of the SLAMs and genes used.

All these markers and genes were combined to create a more complete physical map, hybridising all of them under the same conditions, and comparing the quality, reproducibility and FIpter (The Fractional length from the p-terminus) distances of the signals from two different probe preparation techniques (DOP-PCR and nick translation) for most of the BAC clones as described in sections 3.3.4 and 3.3.5. Finally, FIpter distances were also compared with four previous physical maps (Boonphakdee, 2005; Cnaani *et al.,*

In review; Harvey *et al.,* 2003b; Martins *et al.,* 2004) to obtain the final version of the partial physical map of the tilapia genome.

3.3.3 Controls and cross reference markers

Each of the BAC clones selected for *in situ* hybridisation were previously identified by Bo-Young Lee and Aimee Howe at the University of New Hampshire (BACs from tilapia's library HCGS-03T1 and HCGS-04T1) and Chuta Boonphakdee (BACs from tilapia's library USTI-02T1) at the University of Stirling using PCR amplifications and sequencing procedures.

BAC clones containing the marker *OniY*227 or UNH995/UNH104 were used as reference probes in most of the cases, using dual hybridisation for quality assurance. These two markers gave strong signals on homologous chromosomes and helped to identify any hybridisation problem not related with probe preparation.

Dual hybridisation was practiced also for cross reference between markers identified on different linkage groups (according to Lee *et al.,* 2005))to determine whether separate linkage groups belong to the same pair of chromosomes or not.

3.3.4 Fluorescent *in situ* hybridisation by DOP-PCR

In Chapter 2 (section 2.8.3), DOP-PCR technique was described solely for blocking purposes. Using the same DOP universal primer sequence, this time it was applied for the preparation of probes. Probes prepared by this method

originated from a construct DNA purified from a BAC clone containing the tilapia genomic fragment of interest.

PCR labelling was done in two steps, using the first PCR programme solely for the degenerate amplification of BAC insert DNA, and the second step for labelling with a specific fluorescent nucleotide, either Biotin-16-dUTP or Digoxigenin-11-dUTP (ROCHE, Germany).

The first PCR programme (degenerate amplification programme) was run in PCR tubes (0.2 ml capacity) using a standard PCR master mix (see Table 3.1). The PCR programme included two stages; the first stage run for 10 amplification cycles and the second stage run for 40 cycles, as described in Table 3.2.

Table 3.1 First master mix composition prepared for the degeneration of selected plasmid DNA by DOP PCR.

Components	Stock Solutions	Per Reaction	PCR volumes (µl)
Water	---	---	8.75
Buffer IV	10×	1×	1.5
$MgCl_2$	25 mM	2.5 mM	1.5
dNTPs	5 mM each	0.2 mM each	0.6
DOP Primer	10 µM	1 µM	1.5
Taq Polymerase	5 U/µl	0.05 U/µl	0.15
BAC DNA	150 ng/µl	150 ng	1.0
TOTAL	---	---	**15.0**

Table 3.2 First programme (DOP-PCR preparation) composed of two stage cycles during degeneration process.

Steps	Cycle Parameters		
Initial Denaturation	1 cycle	94 °C	180 seconds
Denaturation Annealing Extension	10 cycles	94°C 30°C 72°C	60 seconds 90 seconds 180 seconds
Denaturation Annealing Extension	35 cycles	95°C 58°C 72°C	60 seconds 60 seconds 180 seconds
Final Extension	1 cycle	72 °C	300 seconds
Hold	---	4°C	---

For labelling purposes, a second PCR reaction programme was applied, using 1 µl of PCR product from the first PCR stage and a modified dNTP master mix with a reduced concentration of dTTP (proportion 5/5/5/4). To prepare 500 µl modified dNTPs master mix, 25 µl from each dATP, dCTP and dGTP, and 20 µl from dTTP (100mM each, original concentration stock) were added to 405 µl of water. Into the PCR master mix was added this time 4 mM/100 µl of either Biotin-16-dUTP or Digoxygenin-11-dUTP (see Table 3.3). The PCR programme was run for 30 cycles as described in Table 3.4.

After the first and second PCR reaction rounds, the resultant amplified fragments were analysed, loading 2 µl of sample in a 1.5% agarose gel and run together with a 100bp ladder (New England BioLabs, UK), expecting to see fragment sizes in the range between 200-1000bp (Figure 3.3).

Additionally, blocking DNA was prepared from male XY Nile tilapia genomic DNA as described in general materials and methods Chapter 2, section 2.8, by DOP PCR. Slide preparation, detection and hybridisation procedures are described in the same section).

Table 3.3 Second master mix composition prepared for the labelling of degenerated plasmid DNA (1µl, from previous PCR reaction) by DOP PCR. Biotin and Digoxygenin fluorescent nucleotides were used in separated reactions using different plasmid sources to complement dual FISH.

Components	Stock Solutions	Per Reaction	PCR volumes (µl)
Molecular Grade Water	---	---	9.25
Buffer IV	10×	1×	1.50
$MgCl_2$	25 mM	2.5 mM	1.50
dNTPs	5/5/5/4 mM each	0.2 mM each*	0.60
Bio or Dig dUTP	1 mM	0.04 mM	0.60
DOP Primer	10 µM	1 µM	1.50
Taq Polymerase	5 U/µl	0.05 U/µl	0.15
Degenerated Plasmid	from previous PCR reaction	---	1.00
TOTAL	---	---	**15.00**

***Modified dNTPs were prepared in a previous master mix including dATP,dCTP,dGTP,dTTP (5/5/5/4mM respectively).**

Table 3.4 Second programme (DOP-PCR) composed of one stage cycles during the label process.

Steps	Cycle Parameters		
Denaturation Annealing Extension	30 cycles	94°C 60°C 72°C	30 seconds 100 seconds 45 seconds
Hold	---	4°C	---

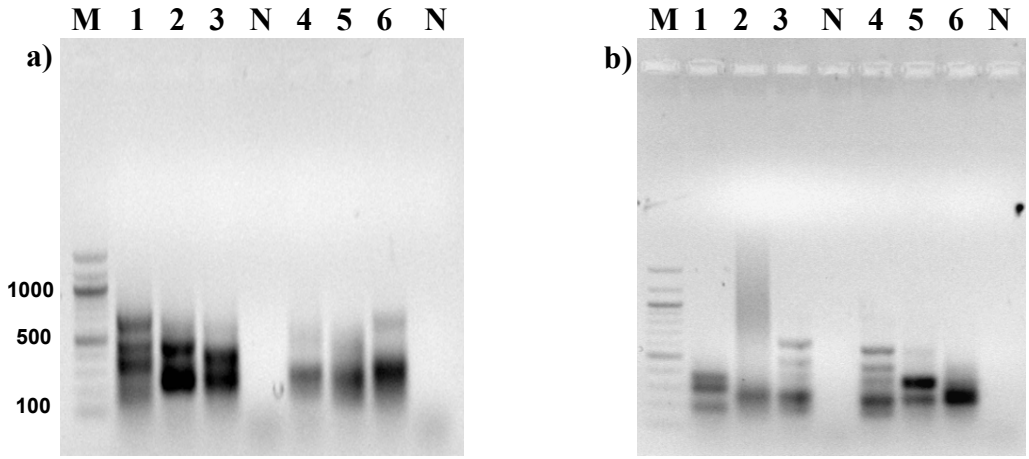

Figure 3.3 Agarose Gel analysis of second stage DOP-PCR products from specific BAC clones. Each lane contains 2µl of second round DOP reaction. M= marker, N= negative control. a) 1 to 3, Insulin, DMT, UNH868 markers labelled with Biotin dUTP; 4 to 6, GM271, GM180, UNH948 markers labelled with Digoxygenin dUTP; b) 1 to 3, UNH115, Trp1, GM128 markers labelled with Biotin dUTP; 4 to 6, GM128, GM150, Trp1pseudogene markers labelled with Digoxygenin dUTP.

3.3.5 Fluorescent *in situ* hybridisation by nick translation

The detailed protocols for preparation of probes by nick translation, blocking DNA and the subsequent hybridisation and detection are described in Chapter 2. Figure 3.4 a) and b) shows labelled constructs and Nile tilapia DOP-PCR amplified blocking DNA respectively.

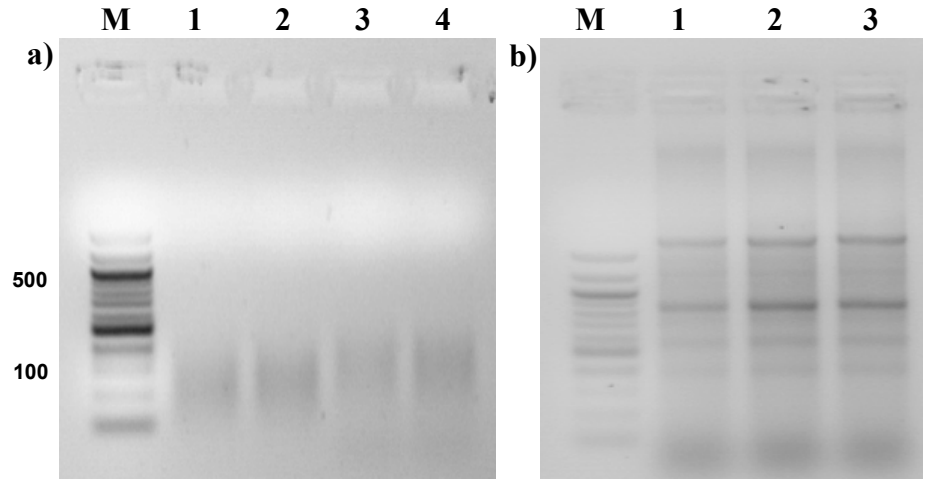

Figure 3.4 3µl BAC Plasmid samples loaded on 1.5% agarose gels showing labelled products by nick translation using 100bp ladder as a marker(M); a) 1 to 4, UNH995/UNH104 (Digoxygenin dUTP), *OniY*227 (Biotin dUTP), DMT/dmrt1 (Digoxygenin dUTP) and UNH995/UNH104 (Biotin dUTP); b) 1 to 3, blocking DNA generate after DOP-PCR procedures.

3.3.6 Fluorescent i*n situ* hybridisation of repetitive satellite DNA sequence (SATA)

This work was done in collaboration with Dr. Cesar Martins from the Universidade Estadual Paulista (Brazil), physically mapping satellite DNA sequences (SATA, previously mapped in *O. niloticus* by Oliveira and Wright, (Oliveira and Wright, 1998) using mitotic spreads prepared by the author from fish stocks held at the University of Stirling.

The physical localisation of Satellite DNA (SATA) was carried out for the species: *O. niloticus, O. aureus, O. mossambicus, O. karongae* and *T. rendalli*; using *O. niloticus* for comparison purposes with the other 4 species.

Satellite sequence (237bp length as single copy; Franck *et al.,* 1992) previously cloned in pUC18 (Oliveira and Wright, 1998) was propagated in *Escherichia coli* strain DH5α (Life Technologies). Fluorescent *in situ* hybridisation was carried according to Pinkel *et al.* (1986), following some

modifications described in Oliveira and Wright (1998) and Martins and Galetti (Martins and Galetti, 2001). SATA probes were labelled by nick translation with biotin-14-dATP (Bionick Labelling System, Gibco-BRL). The slides were incubated in 2× SSC at 37°C for 1h and dehydrated in an ethanol series (75%, 80% and 95%) for 3 min/each. The hybridisation mixture was composed of 50% formamide in 2× SSC, 10% dextran sulphate, 500 µg/ml carrier RNA and 1.5 µg/ml biotinylated DNA probe. After 10 min denaturation at 75°C, 15µl of hybridisation mixture was applied to each slide under a sealed cover slip. Hybridisation was carried overnight in a moist chamber at 37°C. The slides were then washed, under two different stringency conditions (37°C or 42°C), twice for 10 min in 2X SSC. Finally, hybridisation was detected with avidin-FITC conjugate (Sigma) followed by two rounds of signal amplification and the chromosomes were counterstained with propidium iodide (PI; 50 µg/ml) mounted with antifade (Biorad). Then, stacked images were analysed according to section 3.3.9.

3.3.7 Fluorescent *in situ* hybridisation of repetitive telomeric sequence $(TTAGGG)_n$

This work was undertaken also in collaboration with Dr. Cesar Martins, who physically mapping the telomeric sequence on mitotic spreads prepared by the author. A telomeric repeat probe was generated by PCR with the primers (TTAGGG)5 and (CCCTAA)5 according to Idjo *et al.* (1991). Probes were labelled with biotin-14-dATP (Bionick Labelling System, Gibco-BRL), controlling the size of the fragments as previously described for nick translation. The average size of the nick translation products was determined by gel

electrophoresis. The slides were incubated and dehydrated as mentioned in the previous section (3.3.6), followed by the same conditions for i*n situ* hybridisation, detection and counterstain. It was used 35% formamide concentration during washing steps.

3.3.8 FIpter measurements

Mitotic chromosomes were studied mainly at metaphase stage of the cell division, but also during prophase (the first stage when only one chromatid is visible) and prometaphase (when for the first time, two chromatids per chromosome are visible and substructures are well defined). Meiotic chromosomes were observed according to Griffin *et al.* (Griffin *et al.,* 2002) at early pachytene (larger bivalent partially unpaired) and late pachytene (almost paired or full paired larger bivalent). In order to compare different stages of chromosome formation and contraction from different chromosome plates at different cell stages, chromosome 1 (the longest and easiest chromosome to recognise in tilapia karyotypes) was used for calculating relative sizes of the autosomal chromosomes. In addition, centromere position in mitotic and meiotic chromosomes were estimated, based on the fractional length from the p-terminus (FIpter).

Relative measurements allowed the construction of a representative karyotype from *O. niloticus* and *O. karongae* not only from mitotic but also from meiotic chromosomes. Moreover, scaled ideograms were designed for both species based on FIpter measurements on chromosome 1.

Picture measurements are represented with a bar scale of 5 μm. WCIF ImageJ ver.1.37b program (http://www.uhnresearch.ca/facilities/wcif/ imagej/) was used to calculate FIpter measurements (measuring linear distances between two indicated points). A stage micrometer (scale 1 mm = 10 μm with accuracy of 1μm +/-; Carl Zeiss, Gottingen, German) was also used for calibration purposes. Pictures were calibrated according to the two magnifications used: 100× and 150×. Visualising chromosomes at 100× magnification, the scale conversion from a picture on RGB format was 123 pixels = 10μm, and at 150× magnification, 181 pixels = 10 μm.

3.3.9 Karyotyping of *O. karongae* from mitotic metaphase spreads

Male *O. karongae* karyotypes were constructed based mainly on DAPI counterstain (giving blue coloured chromosomes at 360 nm wavelength) for visualisation of chromosomes, and secondly on Cy3 (red colour for specific marker hybridisations with a wavelength of 568nm) and FITC (green colour for specific marker hybridisations with a wavelength of 488 nm) observations. From each chromosome plate, three separate digitised pictures were captured using different wavelength filters.Using CytoVision 2.7 software (Applied Imaging International, UK). DAPI, Cy3 and FITC fluorescence were then stacked on a single image.

Choosing from more than 20 stacked images, three of the best chromosome spreads (with complete set of chromosomes 2n=38 and clear morphology) were chosen for the construction of a model karyotype. From the selected images, only DAPI layers were used. These were converted into black

(chromosomes) and white (background) pictures with Microsoft Office Picture Manager Software (2003) for easier visualisation. Microsoft paint ver. 5.1 was used to cut and sort individual chromosome images and WCIF ImageJ ver. 1.37b program for FIpter chromosome measurements.

From another set of 22 stacked pictures generated from SATA hybridisations (see section 3.3.6) stained with propidium iodide (giving a red fluorescent background at 617 nm wavelength), three of the best chromosome spreads were also selected for the construction of a male *O. karongae* karyotype. This time, the karyotype was constructed using the stacked original red (chromosomes) and black (background) coloured images.

Chromosome pairs were identified using FIpter relative size, centromeric staining, chromosome arm ratios, hybridisation of markers and centromere SATA probes. Chromosomes were arranged from the largest to the smallest and when similar sizes, from the metacentric to the telocentric chromosome (according to the International System for Human Cytogenetic Nomenclature, (ISCN, 1995).

Finally, a comparison of DAPI and PI karyotypes was made for checking any possible inconsistencies in the positions assigned to chromosomes.

3.3.10 Karyotyping of *O. niloticus* and *O. karongae* from meiotic SC spreads

Meiotic karyotypes from *O. niloticus* and *O. karongae* were constructed based on DAPI counterstain (giving blue coloured chromosomes at 360 nm wavelength) for visualisation of chromosomes and in some of the cases Cy3

and FITC fluorescent signals. Similar to the procedure followed in the previous section (3.3.9), from about 20 images from each of the two species, 3 of the best quality pictures were selected for the construction of the model karyotypes. Taking the DAPI layer image, they were turned into black (chromosomes) and white (background) pictures. The same software programmes were used as before for sorting and measuring of the chromosomes, applying this time a straightening tool from WCIF ImageJ ver. 1.37b program to assist during the arrangement of chromosomes.

Chromosome pairs were identified using FIpter relative size, centromeric staining, hybridisation of markers by FISH techniques and unpaired region in the case of *O. niloticus.* Chromosomes were then arranged from the largest to the smallest and when of similar size, from the metacentric to the telocentric chromosomes (according to the International System for Human Cytogenetic Nomenclature (ISCN, 1995).

During the process of karyotyping, a standard and accurate chromosome nomenclature relating FIpter to chromosome types (Figure 3.5) was employed (Czepulkowski, 2001; ISCN, 1995; Tamarin, 2002; Winter *et al.,* 2002) avoiding confusions in the way the chromosomes were named across the karyotypes and different species used in this study.

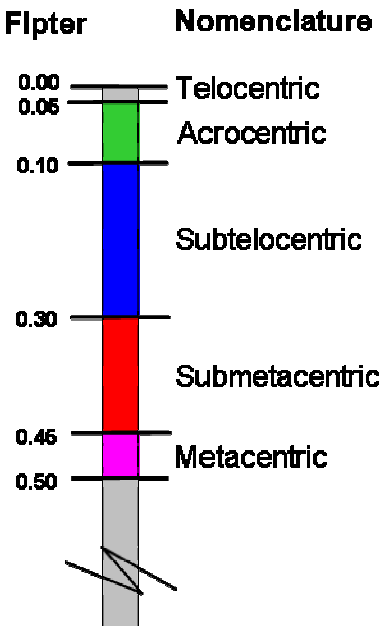

Figure 3.5 Ideogram representing centromere range positions along the chromosome (in FIpter) and their respective nomenclature.

3.3.11 Assignment of tilapia linkage map to chromosomes

BAC probes hybridising to chromosomes, were categorised according to the strength of their signal as strong, medium and weak (see Appendix VI, VIII, IX and X), taking as a reference the strong signal obtained from *OniY*227 BAC clone. Weak signals usually referred to a dispersed and diffuse signal.

As mentioned in section 3.3.2, BAC inserts containing markers or genes belonging to LG1, LG3, LG6, LG7, LG10, LG12 were localised to their respective chromosome and physical distances (mitotic FIpter) between BAC markers were compared with their recombination distances (centimorgans, cM) within their linkage groups. Only the FIpter distances from the marker *OniY*227, the gene DMO (dmrt4), the centromere and the unpaired region from chromosome 1, hybridised onto meiotic chromosomes, were converted into cM distances for a direct comparison with LG3 map, using the formula:

cM= 95-(Flpter X 95)

Where 95 is the total length in cM of the LG3 map (Lee *et al.,* 2005).

Map comparisons of chromosome 1 and other chromosomes lead to the identification of any contraction or expansion of the LGs with respect to the physical distances of genes and markers, to the orientation of LGs respect to their chromosomes and to the anchoring of the existing LGs in tilapia with new markers and genes.

Some other comparisons were also made as mentioned in section 3.3.2 with previously designed physical maps in order to get a more representative and objective physical map from tilapia.

3.4 Results

3.4.1 Mitotic Studies

3.4.1.1 Summary of FISH results from mitotic spreads

A list of the BAC clones physically mapped showing consistency in their signals is given in Table 3.5 (by DOP-PCR) and Table 3.6 (by nick translation) using DOP-PCR and nick translation respectively, for labelling of probes.

Both DOP-PCR and nick translation probe preparations were compared mainly for the BACs which hybridised to chromosome 1. DOP-PCR probes showed high affinity to hybridise on chromosome 1 (more specifically on the distal part of the q arm) irrespective of the linkage group the probe originated from, and even using higher levels of stringency (reducing SSC concentration from 2X to 1X and 0.1X during washing steps). Only BAC clones identified by

the University of New Hampshire were prepared using DOP-PCR (except the SLAM *OniY*227 that served as a positive control during dual hybridisation). The BAC clones identified by the University of Stirling were omitted from this study since they had been mapped before using DOP-PCR for probe preparation (Boonphakdee, 2005; Harvey *et al.,* 2003b). Consistent hybridisation signals were recognised by comparing several images and increasing or decreasing the threshold of the signal (this action also increases or decreases the background signal of the image) before stacking the final image. By DOP-PCR, 7 microsatellites, 1 AFLP and 5 genes showed consistency. They were distributed on chromosomes 1, 2 and one small autosome. Their relative positions are represented in the ideograms from Figure 3.8, 3.13 and 3.15 respectively. BAC clones 6T and 7T both contained the marker GM354, but their hybridisation signal location were reassured to be slightly different (0.80 and 0.76 respectively), so an intermediate point at Flpter 0.78 was taken for its graphic representation.

Labelling of probes by nick translation gave clearer results (better reproducibility, brightness and sharpness) for most of the BAC clones physically mapped (17 out of 24 markers) except for 2T, 11T, 15T, 28T, Sox9bC, InsulinC and 420/425C (GM271, clcn5, UNH115, GM150, Sox9b, Insulin and *OniXY*420/425 respectively) that gave two hybridisation signals at different locations (along the same chromosome arm or another non-homologous chromosome) with the same, higher or lower intensity (see Appendix VII). The frequency of double signals was consistent for 2T (2 signals on chromosome 1, distal part of q arm), Sox9bC (1 signal on chromosome 1 and another on a small autosome) and 420/425C (1 signal on chromosome 1 and another on

chromosome 2) BACs. Despite the BAC clone 28T (GM150) showing background signals on several small chromosomes, the signal on chromosome 1 was consistent. The BAC clones dmrt1,2,3C (Cluster gene dmrt1,2,3) and 22T (UNH868) failed to give any specific signals at all.

Table 3.5 Selection of markers and genes physically mapped on Nile tilapia *O. niloticus* using probes prepared by DOP-PCR. These BAC clones gave the most consistent signals. BACs listed according to linkage group.

***O. niloticus* FISH (DOP-PCR)**						
LG	**BAC Clone no. ***	**Marker/Gene**	**Physical Map**	**FIpter Centromere**	**Chrom. Type (Mitosis)**	**FIpter Mean (±SD)****
1	13T	Aromatase (ovary)	Small Chrom.	0.30	Subtelocentric/ Submetacentric	0.37
3	2T	GM271	Chrom. 1	0.14	Subtelocentric	0.83 (±0.03)
3	4T	GM204	Chrom. 1	0.14	Subtelocentric	0.66 (±0.09)
3	6T	GM354	Chrom. 1	0.14	Subtelocentric	0.80
3	7T	GM354	Chrom. 1	0.14	Subtelocentric	0.76
3	9T	DMO(dmrt4?)	Chrom. 1	0.14	Subtelocentric	0.25
3	10T	DMO(dmrt4?)	Chrom. 1	0.14	Subtelocentric	0.24
3	11T	CLCn5	Chrom. 1	0.14	Subtelocentric	0.35
3	14T	GM180	Chrom. 1	0.14	Subtelocentric	0.46
3	15T	UNH115	Chrom. 1	0.14	Subtelocentric	0.75
3	27T	GM128	Chrom. 1	0.14	Subtelocentric	0.61
3	28T	GM150	Chrom. 1	0.14	Subtelocentric	0.22
3	29T	Trp1 pseudogene	Chrom. 1	0.14	Subtelocentric	0.41 (±0.18)
3	227C	*OniY*227	Chrom. 1	0.14	Subtelocentric	0.56
7	12T	Wt1a	Chrom. 2	0.19	Subtelocentric	0.25

* T = BAC clones identified by Bo-Young Lee and Aimee Howe (Univ. New Hampshire)
C = BAC clones identified by Chuta Boonphakdee (Univ. Stirling)

** ±SD are presented where available

Table 3.6 Selection of markers and genes physically mapped on Nile tilapia *O. niloticus* using probes prepared by nick translation. These BAC clones gave the most consistent signals. BACs listed according to linkage group.

***O. niloticus* FISH (Nick Translation)**						
LG	**BAC Clone no. ***	**Marker/Gene**	**Physical Map**	**FIpter Centromere**	**Chrom. Type (Mitosis)**	**FIpter Mean (±SD)****
1	17T	UNH995 & UNH104	Small Chrom.	0.30	Upper border Subtelocentric	0.67 (±0.09)
3	2T	GM271	Chrom. 1	0.14	Subtelocentric	0.85 (±0.04)
						0.75 (±0.06)
3	6T	GM354	Chrom. 1	0.14	Subtelocentric	0.81 (±0.04)
3	11T	CLCn5	Chrom. 1	0.14	Subtelocentric	0.34 (±0.03)
3	15T	UNH115	Chrom. 1	0.14	Subtelocentric	0.47 (±0.05)
3	25T	Trp1	Chrom. 1	0.14	Subtelocentric	0.20 (±0.04)
3	26T	GM128	Chrom. 1	0.14	Subtelocentric	0.21 (±0.05)
3	28T	GM150	Chrom. 1	0.14	Subtelocentric	0.22
3	dmrt4C	DMO (dmrt4)	Chrom. 1	0.14	Subtelocentric	0.25 (±0.05)
3?	Sox9bC	Sox9b	Chrom. 1	0.14	Subtelocentric	0.56 (±0.07)
			Small Chrom.	0.23	Subtelocentric	0.41 (±0.12)
3?	InsulinC	Insulin	Chrom. 1?	0.14	Subtelocentric	0.70 (±0.10)
3	227C	*OniY*227	Chrom. 1	0.14	Subtelocentric	0.57 (±0.04)
3	InsulinC	*OniY*382	Chrom. 1	0.14	Subtelocentric	0.70 (±0.10)
3 7?	420/425C	*OniX*420 & *OniY*425	Chrom. 1	0.14	Subtelocentric	0.49
			Chrom. 2?	0.19	Subtelocentric	0.66 (±0.06)
6	23T	UNH918	Small Chrom.	0.28	Subtelocentric	0.67
6	24T	UNH948	Small Chrom.	0.28	Subtelocentric	0.55
7	12T	Wt1a	Chrom. 2	0.19	Subtelocentric	0.13
7	BrainaromS	Aromatase (brain)	Chrom. 2	0.19	Subtelocentric	0.77 (±0.07)
10	16T	Insulin	Small Chrom.	0.24	Subtelocentric	0.77 (±0.07)
10	31T	GM561	Small Chrom.	0.24	Subtelocentric	0.78
10	32T	UNH915	Small Chrom.	0.24	Subtelocentric	0.60 (±0.08)
12	8T	DMT (dmrt1)	Small Chrom.	0.27	Subtelocentric	0.85 (±0.11)

* T = BAC clones identified by Bo-Young Lee and Aimee Howe (Univ. New Hampshire)
C = BAC clones identified by Chuta Boonphakdee (Univ. Stirling)
S = BAC clones identified by Simon Harvey (Univ. Stirling)
** ±SD are presented where available

The relative position of the centromere was taken for each of the chromosomes hybridising the markers above. Dual hybridisation showed that LG1, LG3, LG6, LG7, LG10 and LG12 belong to different subtelocentric chromosomes, categorised according to the ideogram on Figure 3.5. LG1 was assigned to a small chromosome with centromere at Flpter 0.30; LG3 was assigned to chromosome 1 with centromere at 0.14; LG6 was assigned to a small chromosome with centromere at 0.28; LG7 was assigned to chromosome 2 with centromere at 0.19; LG10 was assigned to a small chromosome with centromere at 0.24; and LG12 was assigned to a small chromosome with centromere at 0.27.

Finally, it is important to note that in the case of getting diffuse or expanded signals on the chromosome, Flpter distances were calculated considering the mid-point of the signal in all the cases.

3.4.1.2 *O. niloticus*; anchoring and assignment of genetic linkage group 3 to chromosome 1

From both list of results shown above, two representations of the chromosome 1 resulted, one showing distribution of probe signals labelled by DOP-PCR and another showing distribution of probe signals labelled by nick translation.

All the markers from this research, previously linked to LG3, hybridised onto the q arm of chromosome 1 despite secondary signals and background in some of the cases as mentioned in the previous section. Markers not previously linked to LG3 were thus integrated into the same physical map on chromosome

1 as was clearly the case for the three SLAMS, *OniY*227 (227C clone), *OniY*382 (InsulinC clone), *OniXY*420/425 (420/425C clone) and possibly SOX9b (SOX9bC clone).

When comparing marker distribution from both labelling techniques it is possible to see that GM354, insulin, *OniY*227, clcn5, dmrt4 and GM150 hybridised at similar positions. GM271, UNH115, GM128 and trp1 (including trp1 pseudogene) by DOP-PCR hybridised on different locations than nick translation differing in the first technique by an Flpter of 0.08, 0.28, 0.36 and 0.17 respectively, towards telomeres on the q arm.

Images from Figure 3.6 give some examples of some of the probes labelled by DOP-PCR where it is possible to see great affinity of probes to hybridise on the distal part of the q arm. Nevertheless, it is possible to observe a constant weak signal in the middle or close to the centromere like in the case of clcn5 gene (11T clone, Figure 3.6, F). On the contrary, images obtained from nick translation probes showed improved specificity and repeatability with less affinity to the distal part of the q arm (see Figure 3.7). At the same time, hybridisation from probes labelled by nick translation made easier the recognition of true signals and allowed more accurate measurements.

Insulin 1 gene was confirmed to be represented in InsulinC clone (BAC library USTI-02TI) and 16T clone (BAC library HCGS-03TI) after sequence comparison of 3.2 Kb from each clone (by Dr. Bill Pohajdak, University of Dalhousie, Canada. Personal communication). Despite their great sequence similarity, InsulinC and 16T clones hybridised on chromosome 1 (Flpter-=0.69, Figure 3.7, C) and on a small autosome (Flpter-=0.76, Figure 3.16, E)

respectively. By reducing the signal threshold, It was possible to see InsulinC clone hybridising on a small chromosome too (weak signal, see Appendix VII). The AFLP marker *OniY*382 was confirmed by sequencing as well (Boonphakdee, 2005) to be present in the InsulinC BAC clone. For comparison purposes with other physical maps, Insulin 1 and *OniY*382 positions are displayed together but with a question mark after Insulin?, with the reserve of having to confirm again the integrity of *OniY*382 sequence into this clone with further analysis.

Even using a high signal threshold, signal from the *OniXY*420/425 marker and Sox9b gene were identified not only on chromosome 1 but also on chromosome 2 and a small chromosome respectively. Figure 3.7, (D) shows an example of *OniXY*420/425 and Figure 3.7, (E) and (F) of Sox9b. In both cases the signal on chromosome 2 and small chromosome were stronger than in chromosome 1, but all of them represented consistent signals. For these particular situations, signal in two different chromosome locations from both BAC clones used (420/425C and Sox9bC) was considered “real”, but unresolved. Because of the previous sex linkage analysis carried with the three SLAMS (*OniXY*420/425, *OniY*227 and *OniY*382) by Mota-Velasco (Mota-Velasco, 2002) and Ezaz *et al.* (2004b) showed close proximity to each other as well as to the sex determining locus, the presence of 420/425C on chromosome 1 was considered more likely than on chromosome 2.

The BAC clone containing the marker GM150 also gave multiple signals on different chromosomes as well as chromosome 1 but the signal on chromosome 1 was the only one showing consistency. “Secondary signals” for instance appeared sometimes on one chromosome 2 and on a small chromosome on the same spread or on two small chromosomes but in different positions with respect to the centromere (background signal). The position of the GM150 marker on chromosome 1 was supported by its association to LG3 and the fact that 10 other markers from LG3 were hybridising on the same chromosome.

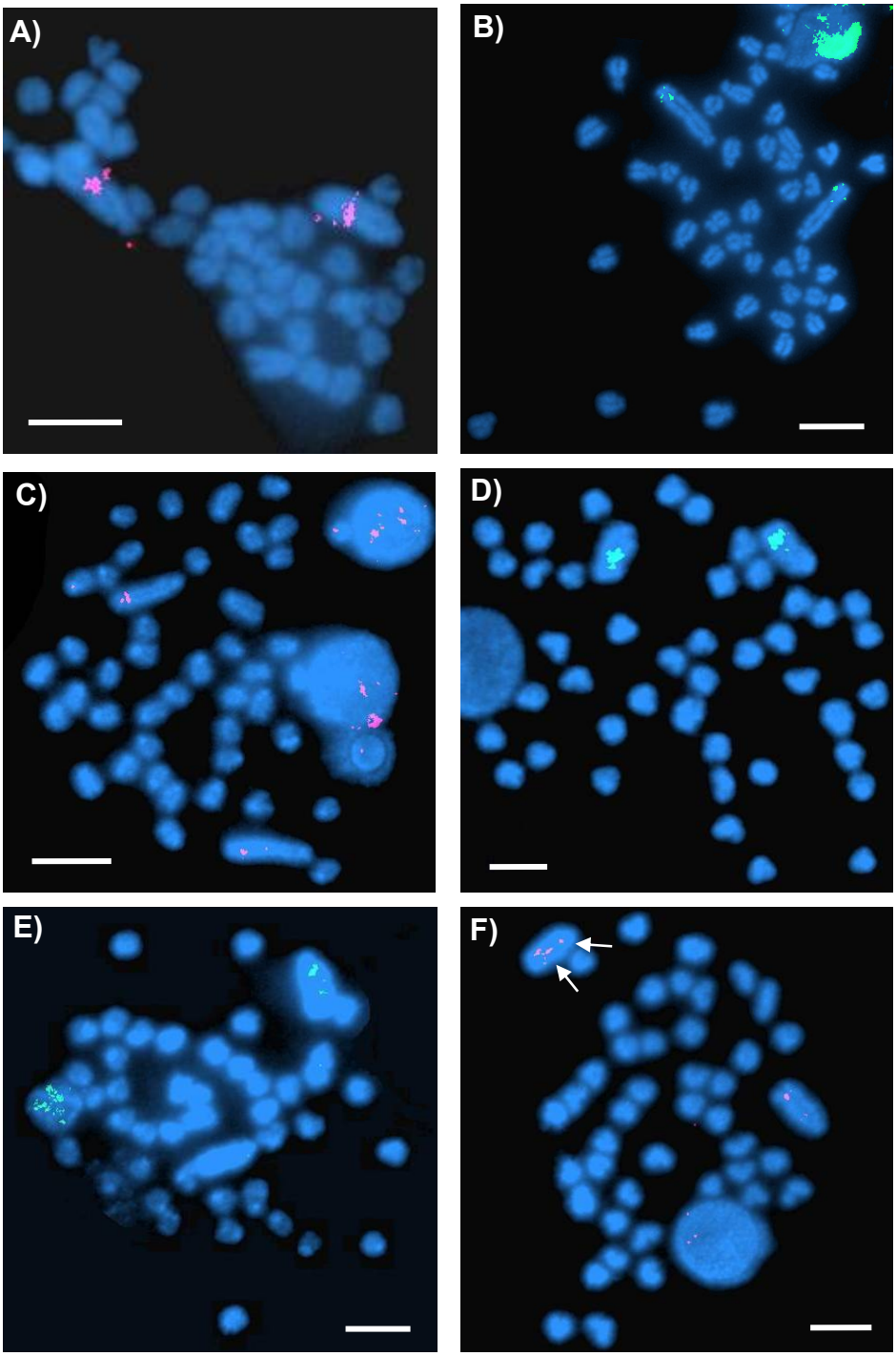

Figure 3.6 Physical mapping of BAC probes on chromosome 1 from *O. niloticus* labelled by DOP-PCR hybridised on single FISH using fluorochromes Cy3 (red fluorescence), FITC (green fluorescence), and DAPI for chromosome counterstain. (A) marker *OniY*227 (227C); (B) marker GM271 (2T); (C) marker UNH354 (6T); (D) marker GM204 (4T); (E) marker dmrt4 (dmrt4C); (F) gene CLCn5 (11T). Scale bar = 5 µm.

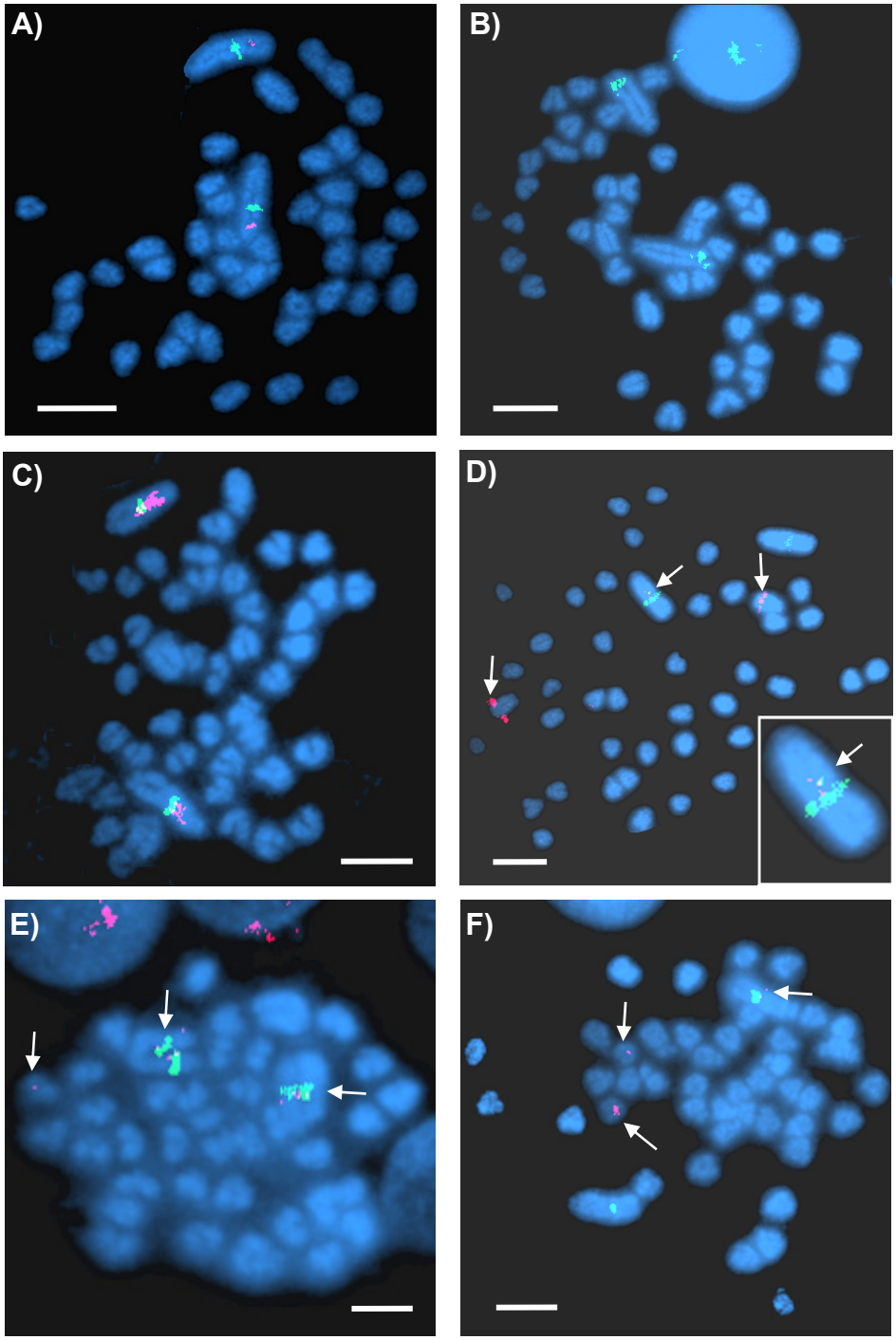

Figure 3.7 Physical mapping of BAC probes on chromosome 1 from *O. niloticus* labelled by nick translation, hybridised on single and double FISH using fluorochromes Cy3 (red fluorescence), FITC (green fluorescence), and DAPI for chromosome counterstain. With exception of picture (B), all pictures show *OniY*227 (227C) in green. (A) marker GM354 (6T); (B) marker GM271 (2T); (C) insulin gene (InsulinC) and marker *OniY*382 (InsulinC), both present in the same clone; (D) marker *OniXY*420/425 (420/425C) with arrows showing in red fluorescence hybridisation on the chromosome 1 and 2; internal picture on the right-bottom amplifies one chromosome 1 with *OniXY*420/425 hybridisation; (E) and (F) Sox9b gene (Sox9bC) with arrows showing in red fluorescence, hybridisation on the chromosome 1 and an autosome. Scale bar = 5 µm.

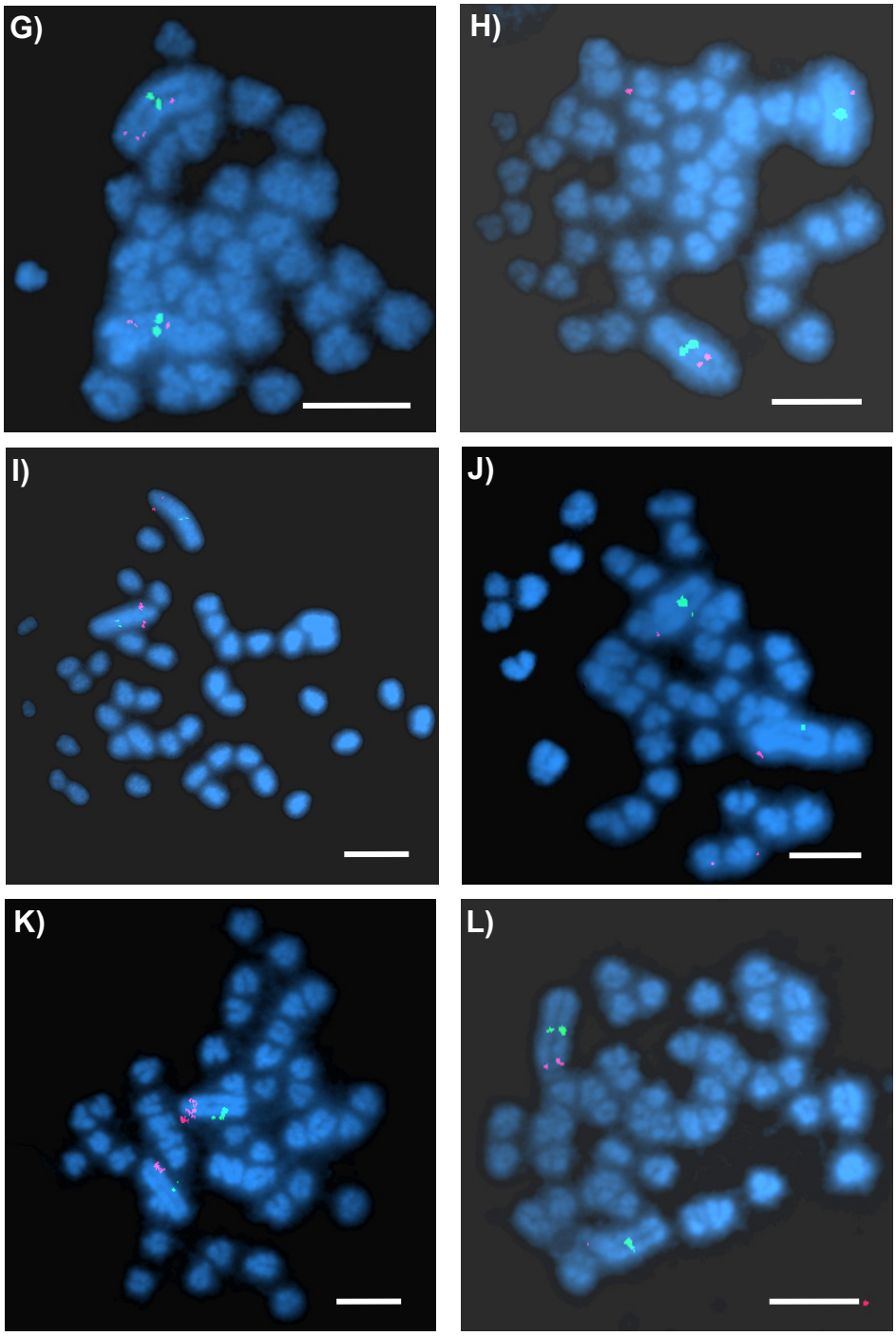

Figure 3.7 (Continued); all pictures show *OniY*227 (227C) in green. (G) marker UNH115 (15T); (H) CLCn5 gene (11T); (I) gene dmrt4 (dmrt4C); (J) marker GM150 (28T); (K) Trp1 gene (25T); (L) marker GM128 (26T). Scale bar = 5 µm.

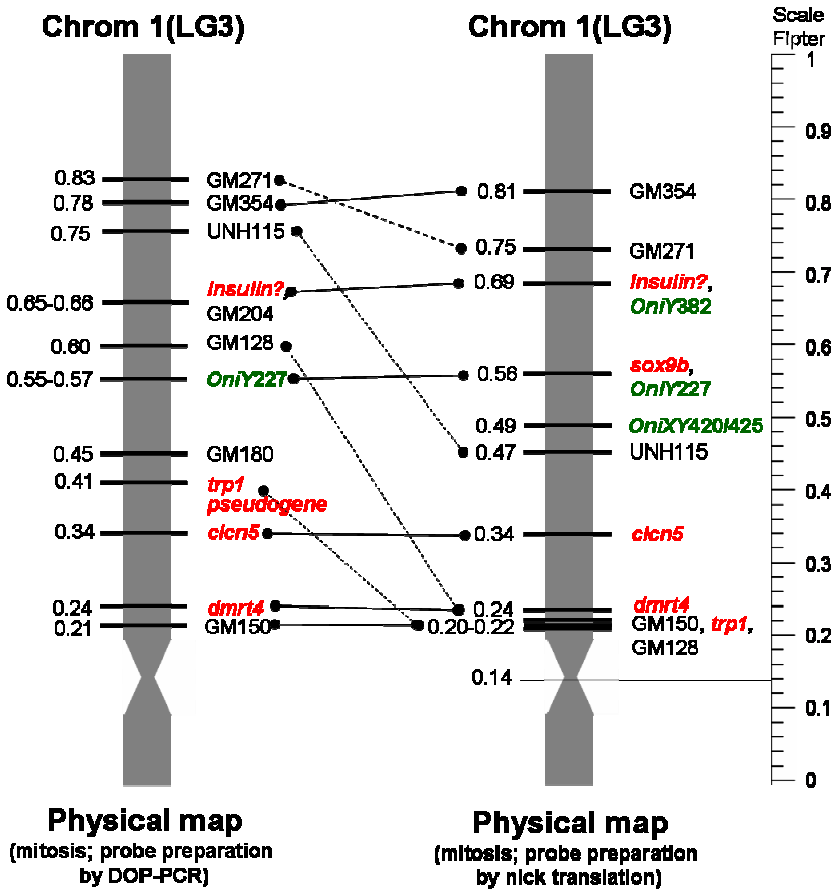

Figure 3.8 Two ideograms representing the physical map of chromosome 1 (LG3) from *O. niloticus* and showing markers and genes distributed along the q arm. A comparison of distributions between DOP-PCR and nick translation probes is made by connective lines; continuous lines connect homologous markers and genes with similar positions, segmented lines also connect homologous markers and genes but with distal positions. Marker positions are in Flpter (scale bar on the right side), showing the location of the centromere at 0.14.

Another set of comparisons was made with previous physical maps (Harvey *et al.,* 2003b; Boonpakdee, 2005) developed from chromosome 1 on mitotic stages, using the cytogenetic map created in this project from probes labelled by nick translation.

Harvey *et al.* (2003b) physically mapped several SINE elements along the long arm of chromosome 1 (1q), while Boonphakdee (Boonphakdee, 2005) localised three SLAM markers developed by Ezaz *et al.* (2004b), insulin gene, 4

copies of the DM family of genes (dmrt 1,2,3 and 4), and 3 copies of the Sox gene (Sox 9b, 11b and 19). These physical maps were created using DOP-PCR probe labelling. When anchoring six common markers (using the same BAC clones from the authors mentioned above) from previous maps with the newly created physical map (see Figure 3.9) it was observed wider and more precise Flpter distribution (rather than just big clusters of markers) for the new map. The insulin gene, *OniY*382, *OniY*227, *OniXY*420/425 and dmrt4 gene showed similar positions. However, the Sox9b gene, moved from a relative position of around Flpter= 0.70 in the previous maps to 0.56 in the new map. The BAC clone containing the cluster gene dmrt1,2,3, could not be mapped under nick translation conditions due to the failure to show specific FISH signal on any chromosome. Only approximate positions were given for this cluster at Flpter= 0.57. Finally, on the previous version map, the centromere was visually estimated with no specific Flpter position.

Cnaani *et al.* (Cnaani *et al.,* In review), developed also a physical map on chromosome 1 using *O. aureus* mitotic chromosomes, using a reduced set of markers to the ones previously mentioned. They described seven markers with a wide distribution along the long arm (1q). When comparing with the physical map developed in the present research using nick translation probe labelling, both maps were anchored with the markers GM354, GM271, UNH115 and Clcn5 gene. These markers were mapped using the same BAC clones for GM354 and Clcn5. The markers GM271 and UNH115 represent BACs with ID b03TI086AF05 and b04TI074AA04 respectively not mentioned before in the present work. In Figure 3.10, the distribution of markers between both physical maps demonstrate conserved order but dissimilar distribution along the long

arm, specially for the markers UNH115 and Clcn5 that were separated by FIpter=0.13 in the present research's map whilst they were mapped together in Cnaani *et al.* (2007) map. On the other hand, GM354 and GM271 are distant in Cnaani *et al.* (2007) map but appeared closer in the present research's map with a distance of FIpter=0.06 in between. Another observation in the distribution was made when comparing GM204 and GM180 markers represented on Cnaani *et al.* (2007) map versus the map made in the present research by DOP-PCR probe labelling (shown in Figure 3.8). These two markers appear in approximately similar location on both maps (e.g. see Figure 3.6, D for GM204 hybridisation). Nevertheless, it has to be considered that the rough positions of Cnaani *et al.* (2007) markers and centromere do not necessarily represent accurate positions (such as FIpter).

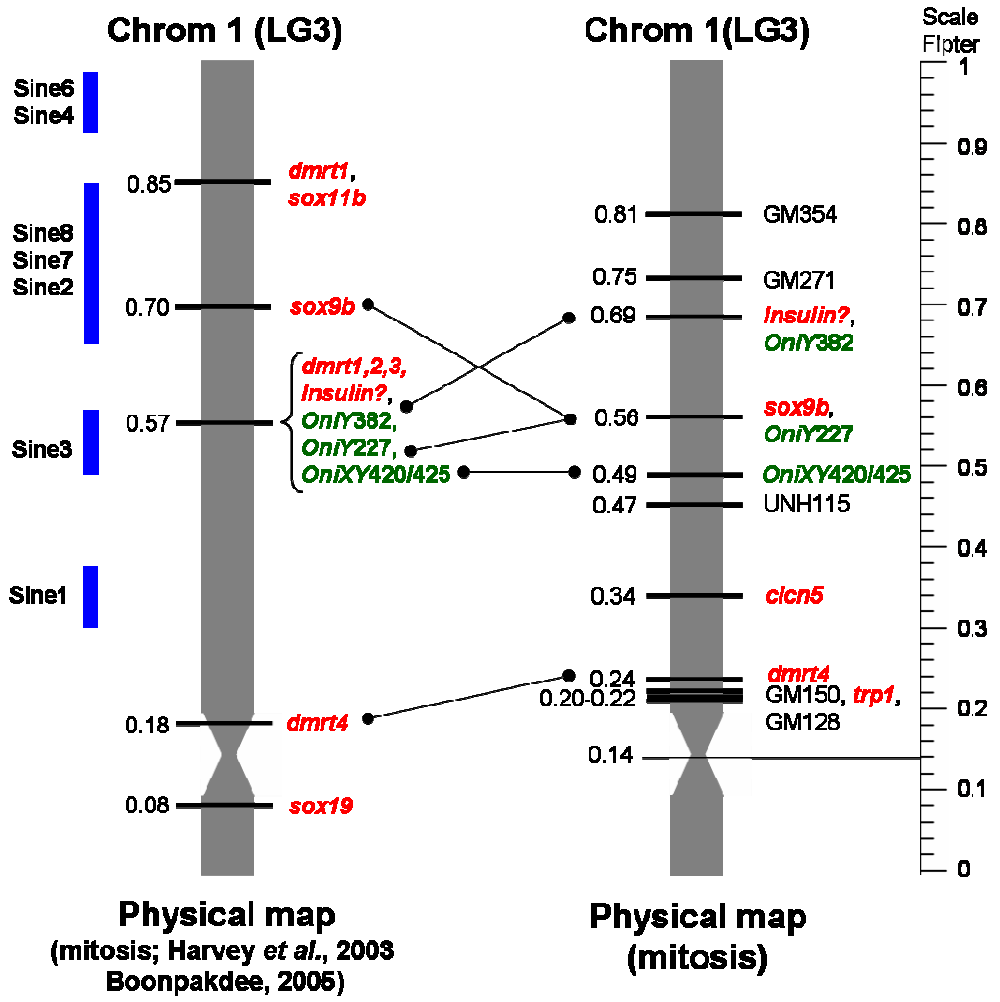

Figure 3.9 Two Ideograms representing chromosome 1 in Nile tilapia *O. niloticus*, showing marker distribution of previous physical maps developed by Harvey *et al.* (2003b) and Boonphakdee (2005) on the left, versus the newly developed physical map nick translation on the right. Connective lines compare common markers between both ideograms. Notice that there were given approximate FIpter positions for markers on the other author's map, so approximate positions are showed. Blue bars on the left represent range positions for SINE sequences. Marker positions are in FIpter (scale FIpter on the right side of ideograms), showing the location of the centromere at 0.14.

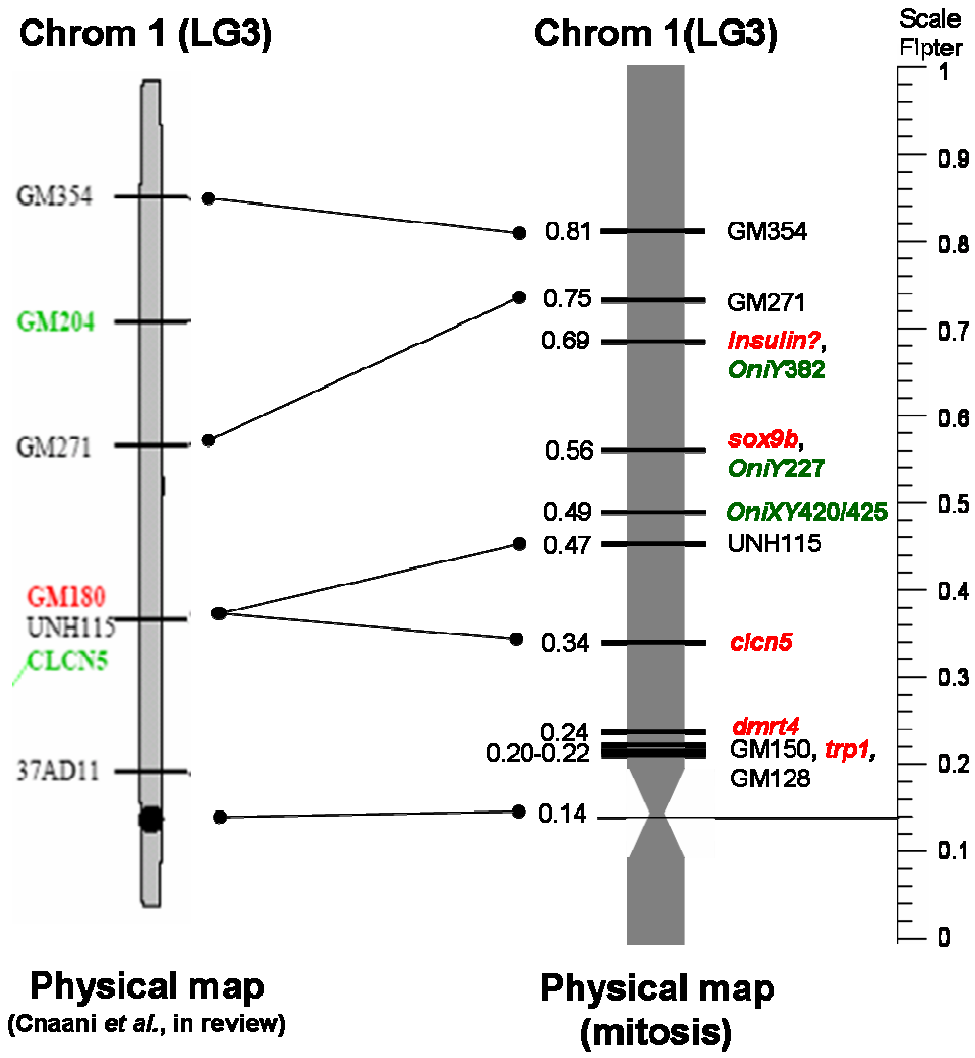

Figure 3.10 Two Ideograms representing chromosome 1 in tilapia, comparing marker distribution of a physical map (on the left) developed by Cnaani *et al.* (2007) in *O. aureus*, versus the newly developed physical map (on the right) nick translation conditions in *O. niloticus*. Notice that Cnaani's map only displays approximate positions of the markers. Connective lines compare common markers between both ideograms. Marker positions are in FIpter (scale bar on the right side), showing the location of the centromere at 0.14.

Finally, at the time of confirming the assignment of LG3 (previously developed by Lee *et al. (*2005) to chromosome 1, the relative distribution of the markers was compared and an integration of new markers into the same LG was proposed. These markers included three SLAMs (*Oni*XY420/425, *Oni*Y227, *Oni*Y382), and two genes (Sox9b and dmrt4) that were physically

located along with five markers (GM354, GM271, UNH115, Gm150 and GM128) and three genes (Clcn5, DMO (dmrt4) and trp1) from LG3 as observed in Figure 3.11. Insulin gene was not considered to be truly on chromosome 1 due to the information from sequence and LG analysis, and a constant secondary signal (with lower signal threshold) on a small chromosome that indicated it belongs to a different linkage group (LG 10) as proposed by Lee *et al.* (2005).

From the comparison of the results, expanded-contracted zones (according to the linear distribution of markers) were observed on LG3 with reference to the physical position of the markers on the long arm (1q) and the linkage distances. As indicated in Figure 3.11, the contracted zone on LG3 was observed from GM354 (0 cM) to Clcn5 (29 cM), a range of 29 cM, distributed on the 47% of the chromosome's total length (1:1.62 rate). The expanded zone from Clcn5 (29 cM) to GM128 (77 cM), a range of 48 cM, distributed on the 14% of the chromosome's total length (3.43:1 rate). This comparison suggests a suppression of recombination in an Flpter range from 0.34 to 0.81 and an abrupt increase in recombination in an Flpter range from 0.20 to 0.34.

In the same figure, the delayed pairing region observed during early pachytene stage on meiotic spreads is displayed in the Flpter range between 0.75 (GM271 physical position) to 1.0 (the far end or telomeres of the q arm). Considering an approximated unpaired origin point at 0.75 and the presence of sex-linked AFLP markers in the proximity, the sex determining region on chromosome one was proposed between the markers GM271 (0.75) and *OniY*227 (0.56). This gives a maximum range of 19% and possibly a minimum

of 6% between GM271 (0.75) and *OniY*382 (0.69) considering the predictions suggested by Ezaz *et al.* (2004b) about the proximity of this *OniY*382 AFLPs to the sex determination locus and the specific suppression of recombination in males on LG3 observed by Lee *et al.* (2004) in this region (see further discussion in section 3.5.3).

BAC clones identified by the Univ. of New Hampshire as red-skin colour related together with observations from Karayucel *et al.* (2004) on red skin colour locus location and a LG created in this project linking red skin colour/black blotches (see Chapter 5), the red skin colour locus was proposed to be in the highly recombinant area between GM128 (0.21) and the centromere (0.14), representing a maximum range of 7% of the total chromosome length where the locus could be physically located.

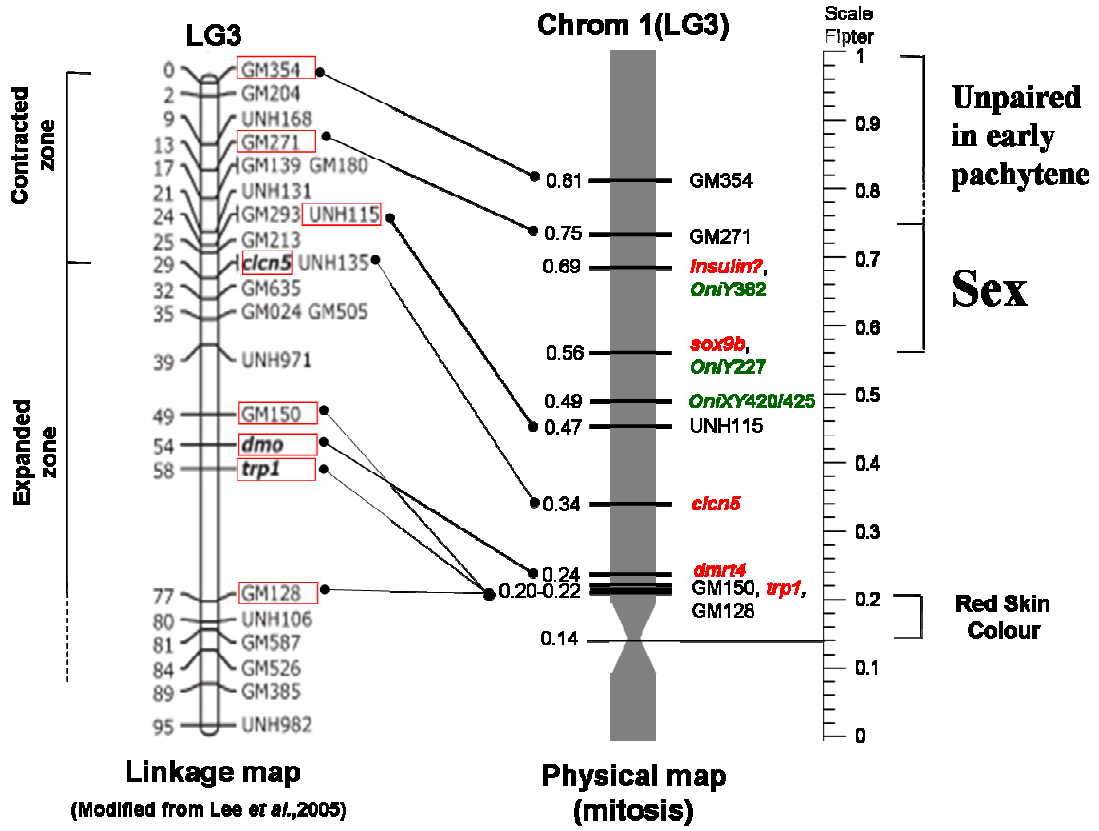

Figure 3.11 Assignment of LG3 (in cM) to the largest chromosome in Nile tilapia *O. niloticus* and integration of a new set of markers and genes. Connecting lines compare common markers between the LG developed by Lee *et al.* (2005), on the left and the mitotic ideogram developed during the present project, on the right. Representative contracted-expanded zones (dot line represents an unknown limit for the expanded zone) are indicated on the far left and the unpaired region observed in early pachytene together with the area proposed for the sex determination and the red skin colour locus, on the far right. Insulin is presented with a "?" due to its hybridisation on a small chromosome too. Marker positions are in Flpter (scale bar on the right side), showing the location of the centromere at 0.14.

3.4.1.3 *O. niloticus*; assignment and anchoring of genetic linkage group 7 to chromosome 2

From the set of markers analysed in this study, the gene Wt1a (12T) belonging to LG7 hybridised on the short arm (2p at Flpter=0.12) close to the centromere from chromosome 2 in the Nile tilapia karyotype. This localisation was obtained by the nick translation labelling technique. However, using DOP-PCR for labelling, the same BAC clone, 12T, hybridised very close to the

centromere on the long arm (2q at Flpter=0.25) possibly because of unnespecific hybridisation. Nevertheless, as was observed with other probes labelled by nick translation, hybridisations were more consistent and clearer by this technique than by DOP-PCR. Hybridisation by nick translation is demonstrated in Figure 3.12, (A). The proposed orientation for this linkage group with respect to chromosome 2 is illustrated in Figure 3.13; although only one marker from LG7 was physically mapped, the proximal position of Wt1a gene to one of the ends of LG7 and its p location on the chromosome, allowed to consider a q/p orientation.

It was mentioned in a previous section that a stronger signal from *OniX*Y420/425 was observed on chromosome 2 than on chromosome 1 (see Figure 3.7, D). This marker was hybridised together in dual hybridisation with brain aromatase gene (BrainaromS) (Figure 3.12, B), showing very close localisation to each other on the long arm of chromosome 2 (2q) at Flpter=0.66 and 0.76 for *OniXY*420/425 and brain aromatase respectively.

Chromosome 2 is one of the two chromosomes in the karyotype of tilapia that are easy to recognise on mitotic spreads because of its medium size. Chromosome 2 was found to represent 50% of the total length of chromosome 1 at methaphase stage (Figure 3.13).

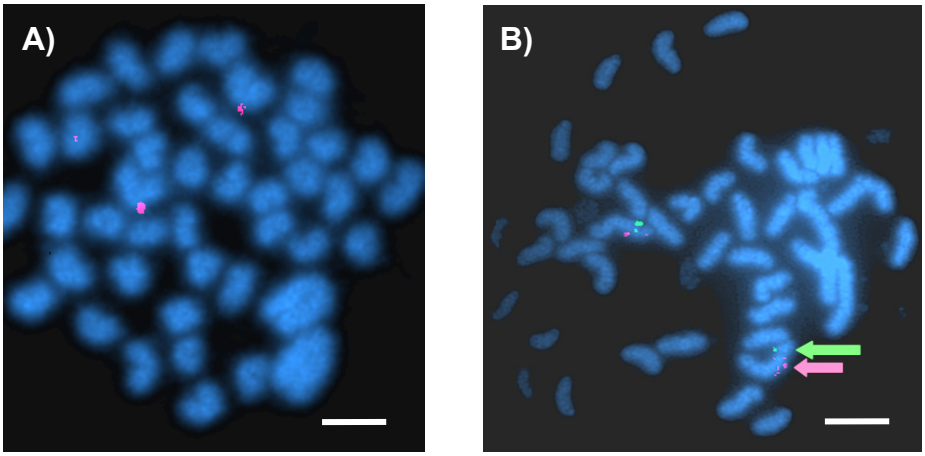

Figure 3.12 Physical mapping of BAC probes on chromosome 2 from *O. niloticus* labelled by nick translation, hybridised by single and double FISH using fluorochromes Cy3 (red fluorescence), FITC (green fluorescence), and DAPI for chromosome counterstain. A) Wt1a gene (12T) from LG7 with a location on the p arm close to the centromere; B) marker *OniXY*420/425 (420/425C) in red colour and brain aromatase (brainaromS) in green colour, indicating positions with coloured arrows on the q arm. Scale bar = 5 µm.

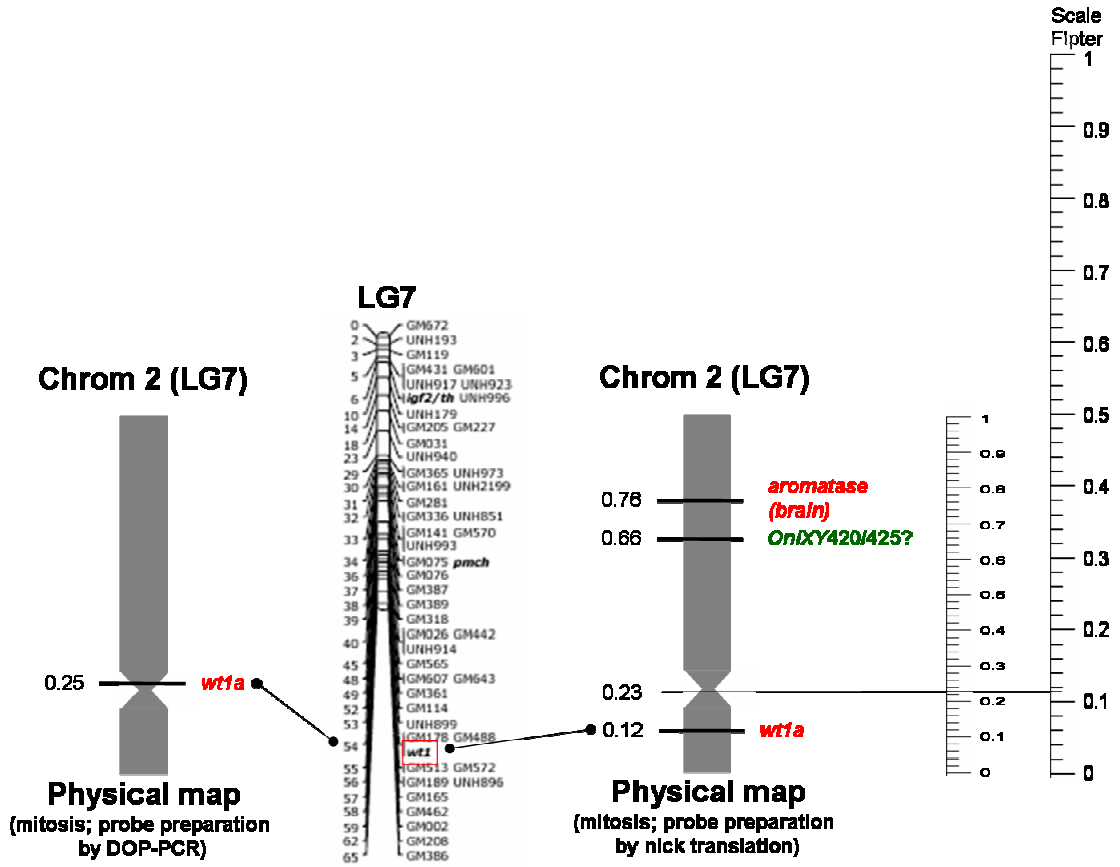

Figure 3.13 Assignment of LG7 to chromosome 2 from *O. niloticus,* showing an orientation of q/p arms according to the LG developed by Lee *et al.* (2005) displayed between both ideograms. Probe signal from Wt1a prepared by DOP-PCR and nick translation is also compared. Scale rulers in Flpter on the right side show proportion of the chromosome 2 respect to chromosome 1. *OniX*Y420/425 is presented with "?" due to its hybridisation on chromosome 1 too.Centromere location is at 0.23 for this chromosome.

3.4.1.4 *O. niloticus*; assignment of genetic linkage groups 1, 6, 10 and 12 to different small chromosomes.

By dual FISH, the assignment of the sex linked (Lee and Kocher, 2007) LG 1 was surprisingly confirmed on a small pair of submetacentric chromosome and not on chromosome 1 as expected (proposed sex chromosome). On LG 1, CyP19A1 or ovarian aromatase (13T) was hybridised on a small chromosome (agreeing with Harvey *et al.,* 2003a) representing about 34% of the total length of chromosome 1 (Figure 3.15). This gene has been included in the tilapia's

linkage map recently by Shirak *et al.* (2006), located at 5cM (between GM314 and GM041) and Lee and Kocher (2007), located at 7.8 cM (between GM633 and GM041). The location of CyP19A1 (Flpter=0.37) on this small chromosome appeared very close to the centromere (Flpter=0.31) on the q arm. Besides, UNH995 and UNH104 that are within the same BAC clone (17T) was localised in the middle of the q arm, helping to orientate the LG (see Figure 3.15). It is important to notice that centromere location (Flpter=0.31) is very close to the border between subtelocentric/submetacentric categories (Figure 3.5), so for the purpose of this report, LG1 will be referred only to be localised on a small pair of submetacentric chromosome even when it could be characterised as subtelocentric in further researches.

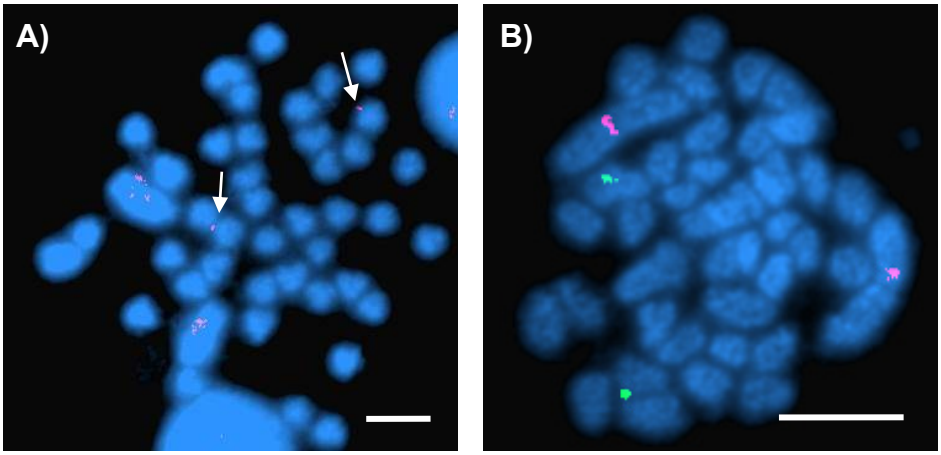

Figure 3.14 Physical mapping of BAC probes on an autosome from *O. niloticus* labelled by nick translation, hybridised on single and double FISH using fluorochromes Cy3 (red fluorescence), FITC (green fluorescence), and DAPI for chromosome counterstain. A) CyP19A1, ovarian aromatase (13T) indicated by arrows on two small autosomes; B) markers UNH995/UNH104 (17T) from LG1 in green colour signals on a q arm of small autosomes and *OniY*227 from LG3 on chromosome 1 in red colour signals for comparison. Scale bar = 5 µm.

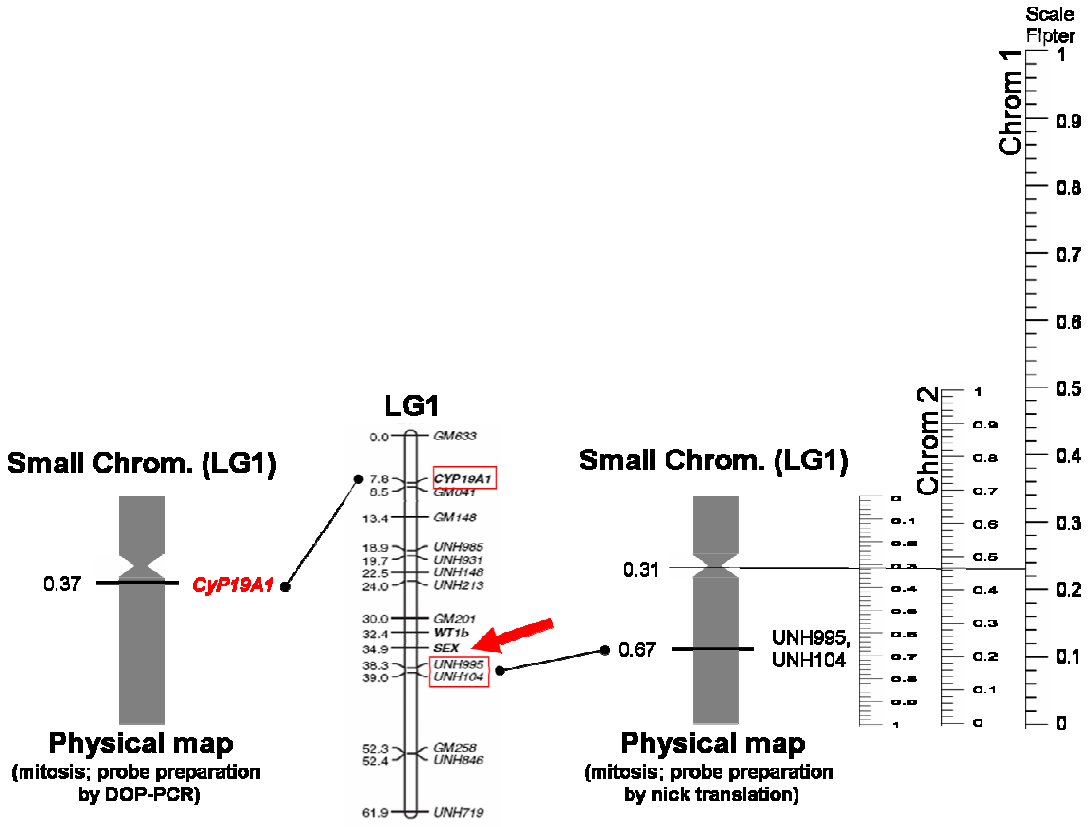

Figure 3.15 Assignment of LG1 to an small submetacentric chromosome pair in *O. niloticus*, showing the orientation of p/q arms according to the LG developed by Lee and Kocher (2007) displayed between both ideograms. Notice that SEX locus, indicated with a red arrow, has been mapped at 34.9cM. Probe from CyP19A1 (ovarian aromatase) was prepared by DOP-PCR and probe from UNH995/UNH104 by nick translation. Scale rulers in Flpter on the right side show the proportion of the small chromosomes (LG1) relative to chromosome 1 and 2. Centromere location is at 0.31 for this small chromosome.

The markers UNH948 (24T) and UNH918 (23T) from LG6 hybridised also to a small subtelocentric autosome, localised around the middle of the q arm at Flpter=0.55 and 0.66 respectively. The centromere was at Flpter=0.28, giving a p/q orientation (the 0 cM end of the linkage group was towards the p arm telomere of the chromosome and the opposite end of the LG towards the q arm telomere) to LG6. LG10 was assigned to a small autosome hybridising markers UNH915 (32T), GM561 (31T) and insulin (16T) with relative positions of 0.60, 0.76 and 0.77 respectively. Centromere position was at Flpter=0.23 for this

subtelocentric chromosome, with an orientation p/q for LG10. Finally, LG12 with the DMT gene (dmrt1, 8T) hybridised to a small subtelocentric chromosome with a proposed orientation of the LG p/q, considering this gene is located near to one of the ends of the linkage groups and hybridising not far from the telomere on the q arm (Flpter=0.85). The centromere is located at Flpter=0.27 in this chromosome. Chromosomes labelled by FISH techniques and linkage groups with their respective ideograms from these three small chromosomes are represented in Figure 3.16 and Figure 3.17 respectively. As mentioned in the beginning of this section, cross checking was made using dual hybridisation to confirm the presence of these four linkage groups on different small chromosomes.

Although the sizes are very close among the small chromosomes during mitotic metaphase, it was observed that LG1 probes hybridised on one of the largest small chromosomes in the karyotype of *O. niloticus*.

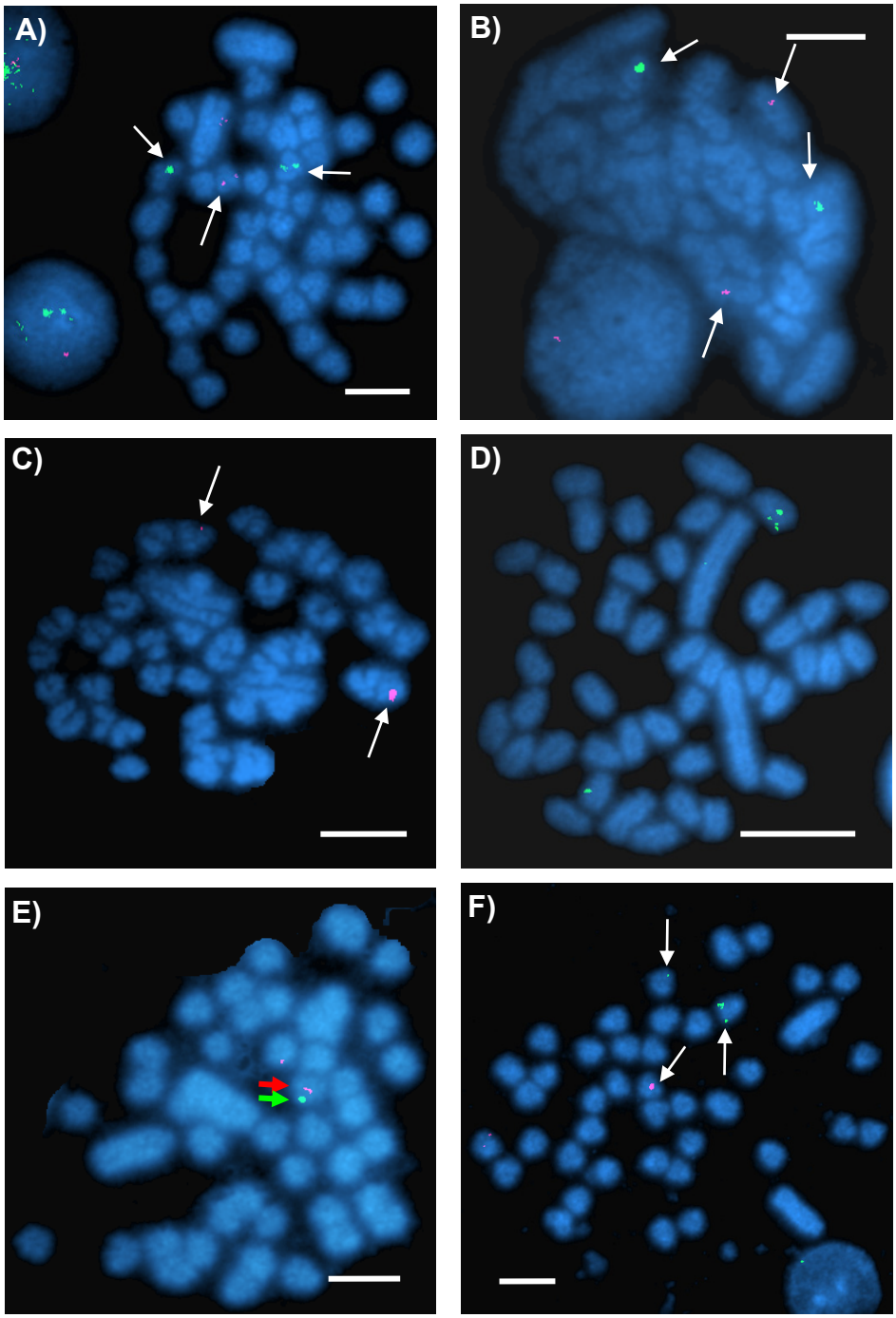

Figure 3.16 Physical mapping of BAC probes on small chromosomes in *O. niloticus* labelled by nick translation, hybridised on a double FISH using fluorochromes Cy3 (red fluorescence), FITC (green fluorescence), and DAPI for chromosome counterstain. A) Marker UNH948 (24T) from LG6 in green colour and UNH915 (32T) from LG10 in red colour indicated by an arrow; notice the separation of both signals in an interphase cell at the bottom-left of picture; B) Marker UNH918 (23T) from LG6 in red colour and markers UNH995/UNH104 (17T) from LG1 in green colour; C) Marker GM561 (31T) from LG10 in red colour indicated by arrows; D) Marker UNH915 (32T) from LG10 in green colour; E) Insulin gene (16T) from LG10 in green colour and marker UNH915 (32T) from LG10 in red colour indicated by two arrows of the same colour; F) DMT gene (dmrt1, 8T) from LG12 in green colour and UNH915 (32T) from LG6 in red colour, both markers indicated by arrows. Scale bar = 5 µm.

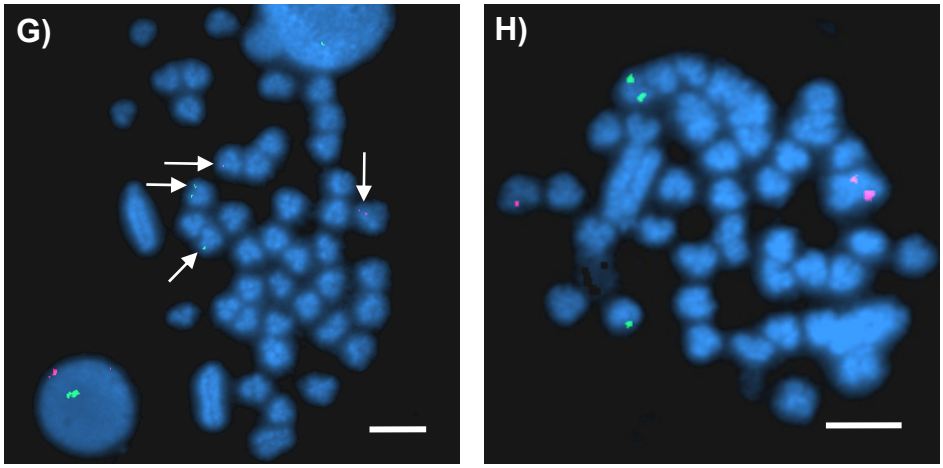

Figure 3.16 (Continued); G) DMT gene (dmrt1, 8T) from LG12 in green colour and marker UNH995/UNH104 (17T) from LG1 in red colour indicated by arrows; notice the separation of both signals in an interphase cell at the bottom-left of picture; H) Marker UNH995/UNH104 (17T) from LG1 in red colour and UNH915 (32T) from LG6 in green colour. Scale bar = 5 µm.

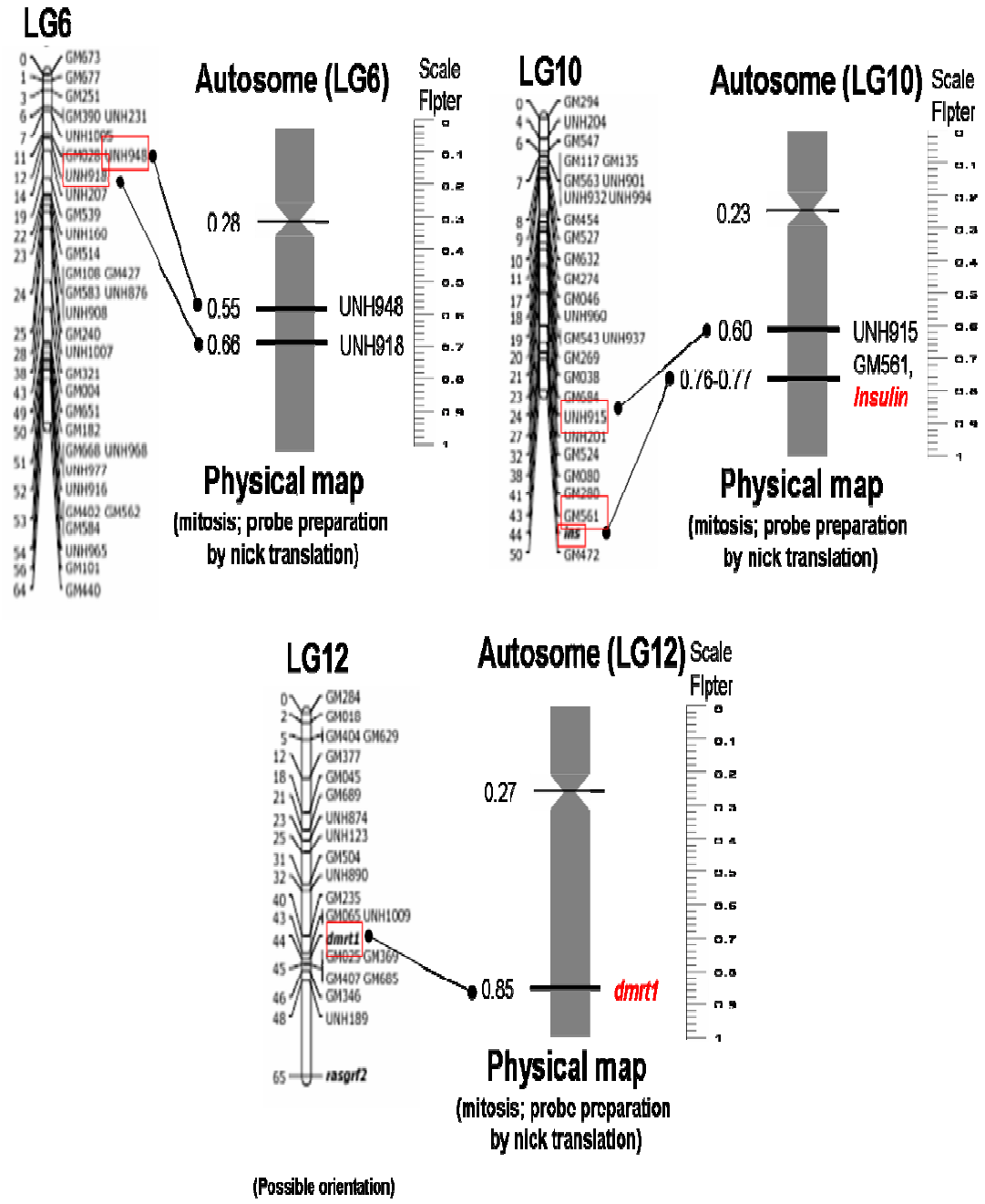

Figure 3.17 Assignment of LG6, LG10 and LG12 to small chromosomes in *O. niloticus*, showing orientations of p/q arms according to their LG developed by Lee *et al.* (2005) displayed on the left side of each ideograms. Orientation of autosome associated with LG12, is only suggested due to the hybridisation of only one marker from its LG. Scale rulers in Flpter on the right side of each ideogram is shown. Centromere locations for small chromosomes associated with LG6, LG10 and LG12 are at 0.28, 0.23 and 0.27 respectively.

3.4.1.5 *O. karongae*; assignment and anchoring of genetic linkage group 1, 3 and 7 to specific chromosomes

In *O. karongae*, the 5 largest chromosomes were characterised with the aid of the specific BAC clones containing markers from LG1, 3 and 7. The marker *OniY*227 (227C, now associated with LG3) hybridised on the q arm of chromosome 1 at FIpter=0.61 and the centromere of this chromosome was at FIpter=0.14. This was very similar to chromosome 1 in *O. niloticus*. The marker UNH995/UNH104 from LG1 hybridised on the q arm of a medium sized chromosome pair. LG7 (Chromosome 2) represented by the markers Wt1a (12T), *OniX*Y420/425? (420/425C) and brain aromatase (BrainaromS) were localised on the p and q arm of another medium sized chromosome pair. *OniX*Y420/425 is considered to belong to chromosome 1 as observed in *O. niloticus* despite a second strongest signal on chromosome 2. In *O. karongae* the strongest signal from the 402/425C BAC clone was observed again on a middle sized chromosome together with the other two markers described before. Metaphase chromosome spreads with representative hybridisations from LG1, LG3 and LG7 probes are shown in Figure 3.18.

Measuring the total length and the centromere position from each of the five largest chromosomes, it was possible to arrange these chromosomes with respect to chromosome 1. In this way, chromosome 2 represented 54% of the chromosome 1 total length and with the centromere at FIpter=0.12 (subtelocentric); chromosome 3 represented 52% with the centromere at FIpter=0.13; chromosome 4 represented 51% with the centromere at FIpter=0.35 (submetacentric); and chromosome 5 represented 49% with the centromere at FIpter=0.24 (subtelocentric). Ideograms representing these

chromosomes and the position of the FISH probes are shown in Figure 3.19. Chromosome pairs 2 and 3, the most similar, can be distinguished by the LG1 FISH probe.

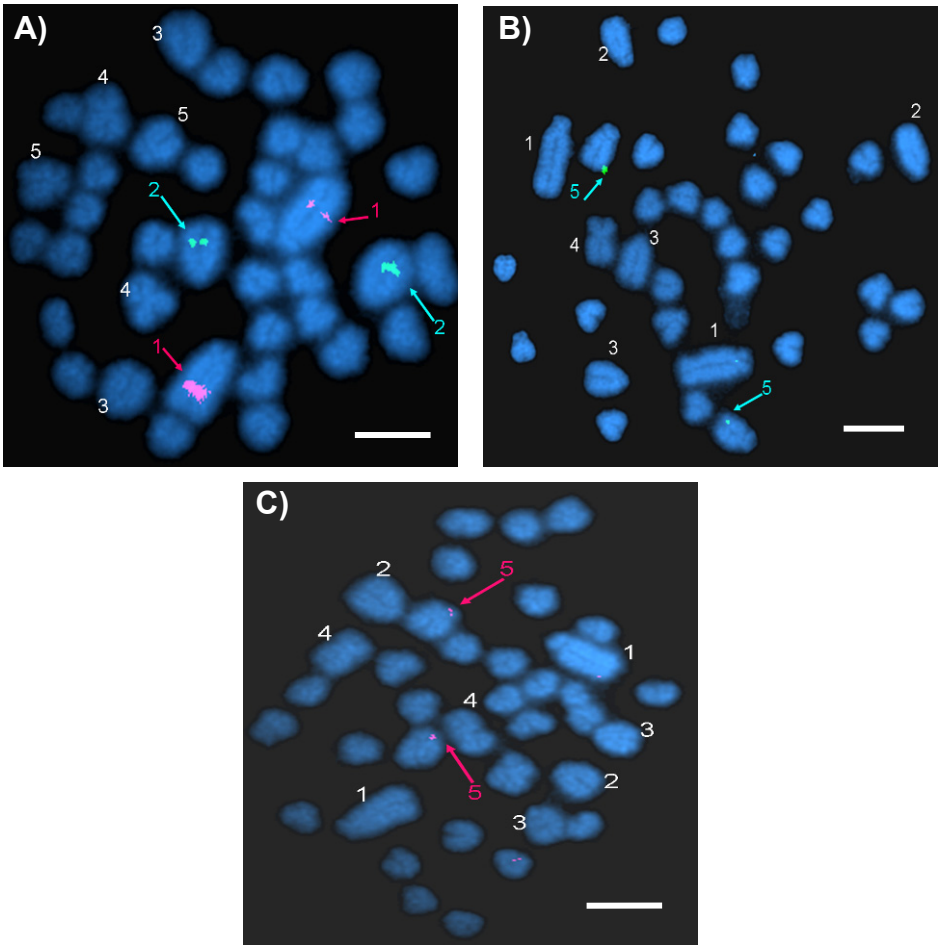

Figure 3.18 Physical mapping of BAC probes labelled by nick translation in *O. karongae*, hybridised on a single and double FISH using fluorochromes Cy3 (red fluorescence), FITC (green fluorescence), and DAPI for chromosome counterstain. The five larger chromosomes in the karyotype are indicated by their respective numbers. A) *OniY*227 (227C) in red colour and UNH995/UNH104 (17T) in green colour; B) Brain aromatase in green colour; C) Wt1a (12T) in red colour. Scale bar = 5 µm.

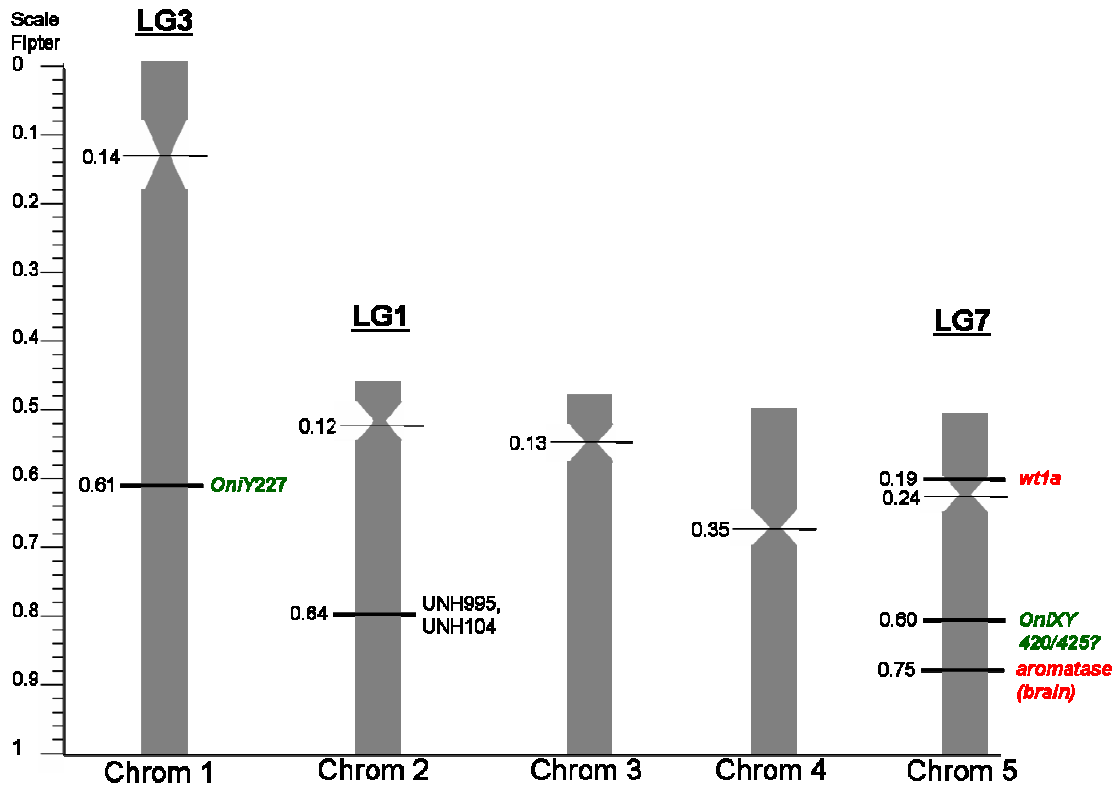

Figure 3.19 Ideograms representing the five largest chromosomes in the karyotype of *O. karongae*. LG3, LG1 and LG7 were assigned to chromosomes 1, 2 and 5 respectively, followed the identification of specific markers on the chromosomes. Scale bar in FIpter is displayed on the left side of chromosome 1 showing the proportions of chromosomes 2, 3, 4 and 5 with respect to chromosome 1, and also characterised by relative centromere positions.

3.4.1.6 *O. karongae*; karyotype rearrangement based on mitotic metaphase spreads

A karyotype arrangement was developed based on mitotic metaphase spreads. Sixteen subtelocentric chromosomes were identified (Chrom. 1, 2, 3, 5, 7, 8, 9, 10, 12, 13, 14, 15, 16, 17, 18, 19) with centromeric FIpter ranging between 0.13-0.28; and 3 submetacentric chromosomes (Chrom. 4, 6, 11) with centromeric FIpter ranging between 0.31-0.35, giving a total of n=19. In Figure 3.20, the karyotype is represented in black and white (originally stained by DAPI). The small chromosomes (6 to 19) showed smaller differences, making

them more difficult to characterise. To aid classification, DAPI stained chromosomes were compared with spreads stained by propidium iodide (PI) and hybridised with SATA sequences to localise centromeres as described later in section 3.4.1.8. There were some difficulties to characterise chromosomes 2 and 3 from the first five larger chromosomes, but combining results from FISH with UNH995/UNH104 (17T), a slight difference was observed in the centromere position or the length of the p arms on chromosome 2, having a lower centromeric Flpter than chromosome 3, as explained in section 3.4.1.5. Further comparisons with the meiotic karyotype in *O. karongae* are made in section 3.4.2.6.

Mitotic Karyotype of *O. karongae*

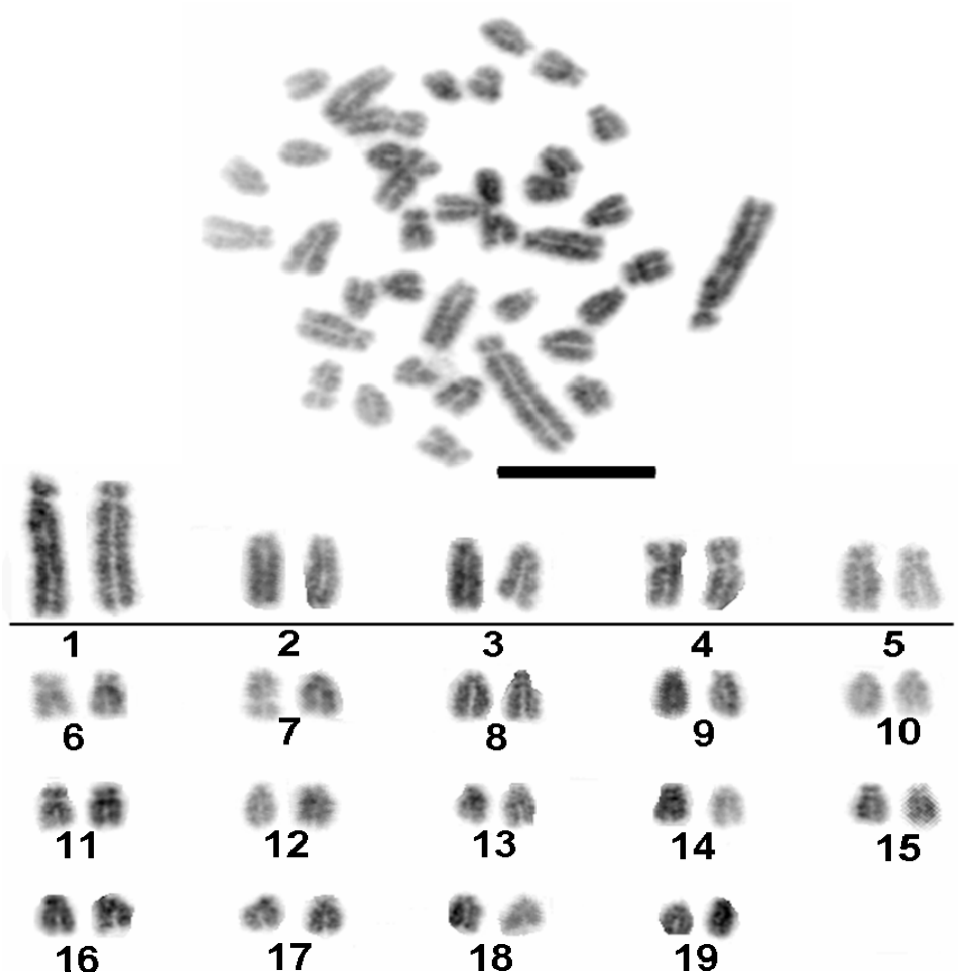

Figure 3.20 Black and white karyotype from a male ZZ *O. karongae* showing the original mitotic metaphase spread and the arranged chromosomes according to total length (descending order) and centromere position (from metacentric to telocentric chromosomes). Original spread was counterstained with DAPI. Scale bar = 5 µm.

3.4.1.7 Other tilapia species; assignment of genetic linkage groups 1 and 3

Metaphase spreads from eight tilapia species were hybridised in single and dual FISH using two sex linked markers, UNH995/UNH104 (17T) and *OniY*227 (227C), belonging to LG1 (small chromosome) and LG3 (chromosome 1) respectively. LG1 marker was found in a pair of small autosomes (around the border of subtelocentric/submetacentric, see Appendix VIII, so for the purposes of this report, following the ideogram in Figure 3.5, LG1 was considered only

submetacentric) in *O. aureus, O. mortimeri, O. mossambicus, O. niloticus, T. rendalli* and *T. zillii*. The exception was *O. karongae,* with LG1 on chromosome 2. The position of LG1 in *S. galilaeus* was not determined due to the poor quality of chromosome preparations. *OniY*227 marker was found always on chromosome 1. No differences in hybridisation pattern were observed between phenotypic males and females from any of the species. This suggests a single fusion event in *O. karongae* only for LG1, considering at the same time that is the only species analysed with a different number of chromosomes (n=19 instead of n=22) compared to the other six species studied. Figure 3.21 shows sex-linked hybridisations in the different species analysed and data collected on Table 3.7.

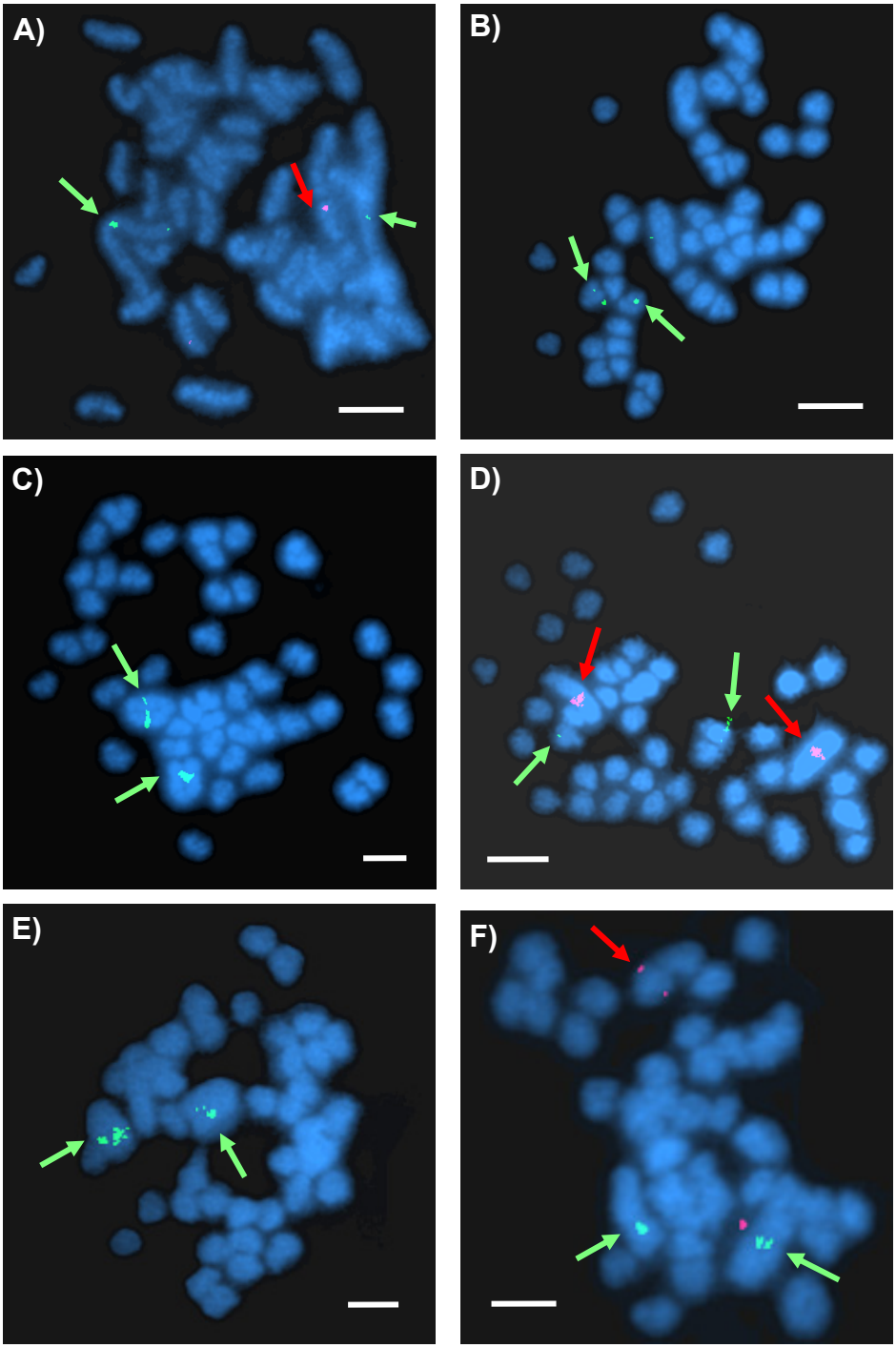

Figure 3.21 Physical mapping of BAC probes labelled by nick translation on several tilapia species, hybridised in single and double FISH using fluorochromes Cy3 (red fluorescence), FITC (green fluorescence) and DAPI for chromosome counterstain. *OniY*227 (227C) from LG3 and UNH995/UNH104 (17T) from LG1. A) *O. aureus* chromosomes with *OniY*227 in green colour and UNH995/UNH104 in red colour; B) *O. mortimeri* chromosomes with UNH995/UNH104; C) *O. mortimeri* chromosomes with *OniY*227; D) *O. mossambicus* with *OniY*227 in red colour and UNH995/UNH104 in green colour; E) *S. galilaeus* with *OniY*227 in green colour; F) *T. rendalli* with *OniY*227 in green colour and UNH995/UNH104 in red colour. Green and Red arrows help to locate their respective signals. Scale bar = 5 µm.

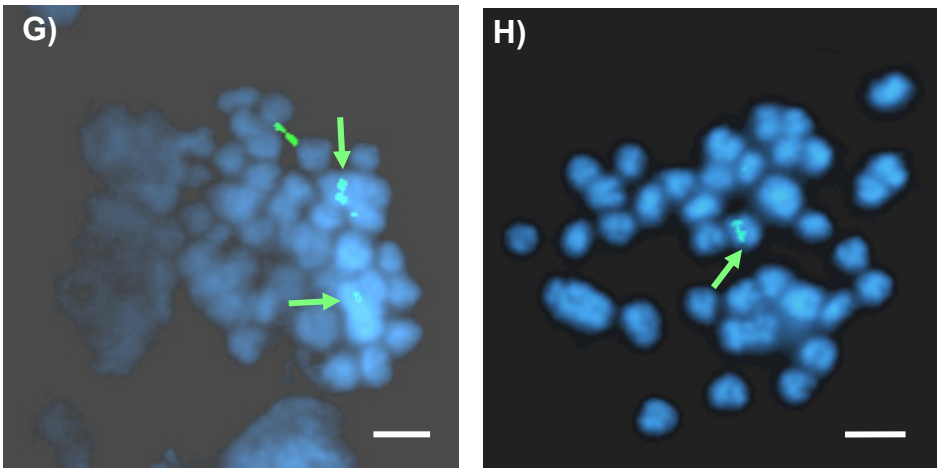

Figure 3.21 (Continued); *T. zillii*, G) marker *OniY*227 (227C) from LG3 on two large chromosomes; H) marker UNH995/UNH104 (17T) from LG1 on two small autosomes. Green arrows help to locate their respective signals. Scale bar = 5 µm.

3.4.1.8 Physical mapping of repetitive satellite DNA sequence (SATA)

Hybridising SATA sequences onto the centromeres by FISH, chromosomes from five tilapia species were preliminary characterised into two general groups of chromosomes according to their visual centromere position (before FIpter measurements) and following the nomenclature described in Figure 3.5: 1. Metacentric and submetacentric chromosomes (M/SM) and 2. Subtelocentric and acrocentric chromosomes (ST/A). Detailed FIpter measurements were only carried later on *O. karongae* for the improvement of the previous karyotype developed with DAPI stain (Figure 3.20).

Table 3.7 Chromosomal distribution of DNA sequences from LG1 (17T) and LG3 (*OniY*227) in selected *Oreochromis*, *Sarotherodon* and *Tilapia* species. Observed haploid chromosome numbers agreed with published data elsewhere (www.fishbase.org).

Species	Haploid chrom. number	LG 1 (UNH995/UNH104)	LG 3 (*OniY*227)	sex determination system (source)	Phenotypic sex of studied animals
Oreochromis aureus	22	Small submetacentric chromosome	Chromosome 1	WZ-ZZ (Mair *et al.*, 1991b)	male
Oreochromis karongae	19	Chromosome 2	Chromosome 1	WZ-ZZ (Cnaani *et al.*, 2007)	LG1 and LG3 in male, only LG1 in female
Oreochromis mortimeri	22	Small submetacentric chromosome	Chromosome 1	XX-XY? (Majumdar, 1986)	female
Oreochromis mossambicus	22	Small submetacentric chromosome	Chromosome 1	XX-XY (Campos-Ramos *et al.*, 2003) or WZ-ZZ? (Cnaani *et al.*, 2007)	male and female
Oreochromis niloticus	22	Small submetacentric chromosome	Chromosome 1	XX-XY (Jalabert *et al.*, 1974)	male and female
Sarotherodon galilaeus	22	?	Chromosome 1	XX-XY? (Majumdar, 1986)	male
Tilapia rendalli	22	Small submetacentric chromosome	Chromosome 1	?	LG1 and LG3 in female, only LG3 in male
Tilapia zillii	22	Small submetacentric chromosome	Chromosome 1	XX-XY (Cnaani *et al.*, 2007)	LG1 and LG3 in male, only LG1 in female

O. niloticus showed 3 pairs of M/SM chromosomes and 19 pairs of ST/A chromosomes; *O. aureus* showed 6 pairs of M/SM chromosomes and 16 pairs of ST/A chromosomes; *O. mossambicus* showed 4 pairs of M/SM chromosomes and 18 pairs of ST/A chromosomes; *T. rendalli* showed 5 pairs of M/SM chromosomes and 17 pairs of ST/A chromosomes and finally, *O. karongae* showed 3 pairs of M/SM chromosomes and 16 pairs of ST/A chromosomes.

Despite having the same number of chromosomes in the first four species mentioned (2n=44), morphology varies, showing different number of M/SM and ST/A for these species. *O. niloticus* and *O. karongae* are the species with the lowest number of M/SM and *O. aureus* the species with the highest number of M/SM. In the case of *O. karongae* (2n=38), there were not only differences in the number of chromosomes but also in the size of the first five chromosomes, as described in the previous section. Figure 3.22 and 3.23 illustrate chromosome arrangement according to visual total length and centromere position.

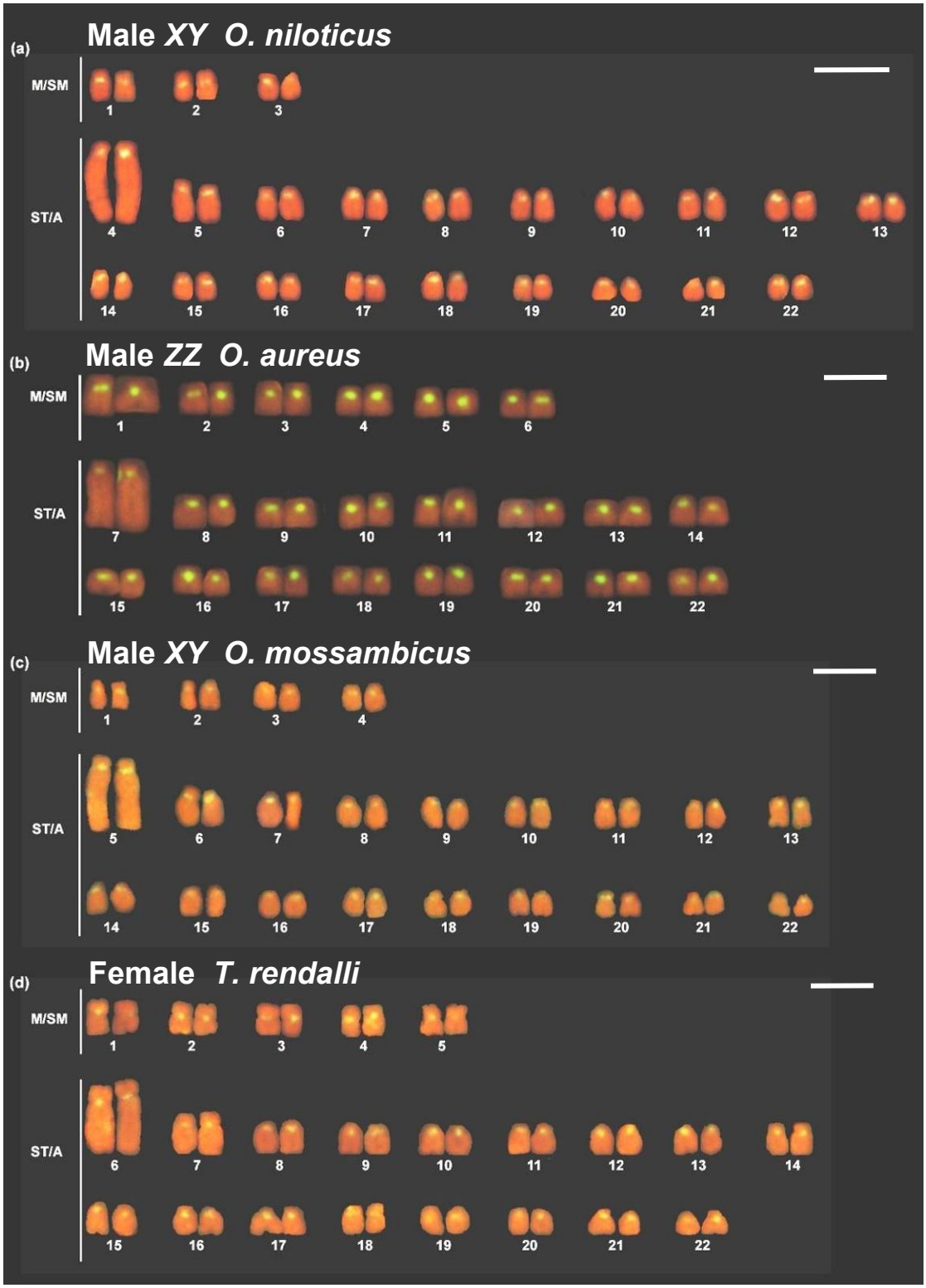

Figure 3.22 Physical map of SATA on different tilapia karyotypes, showing centromere locations of each chromosome. SATA probes were labelled with Biotin-14-dATP and counterstained with propidium iodide. Chromosomes were classified in two groups: M/SM = Metacentric or Submetacentric, ST/A = Subtelocentric or Acrocentric. Scale bar = 5 µm.

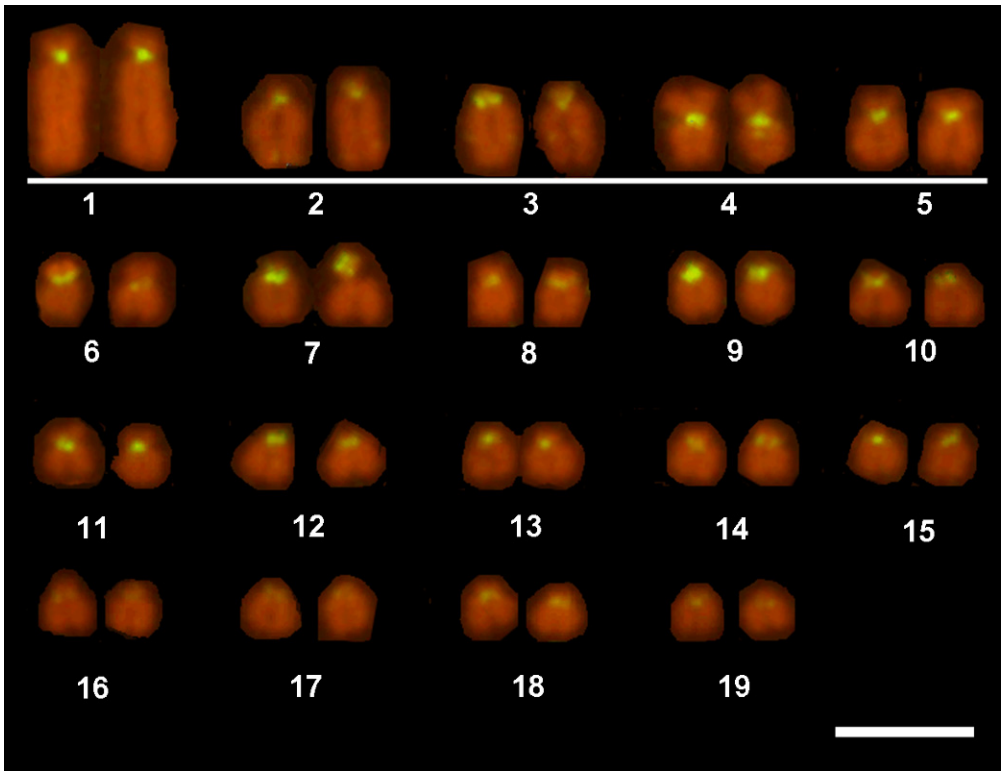

Figure 3.23 Karyotype from a male ZZ *O. karongae* according to the hybridisations obtained by SATA sequences. SATA probes were labelled with Biotin-14-dATP and counterstained with propidium iodide. Original mitotic spread is showed on Figure 3.24, (A). Scale bar = 5 µm.

Based on the *O. karongae* karyotype made from chromosomes stained by DAPI and from others stained by PI with centromeres indicated by SATA, an ideogram of 19 different chromosomes was made (Figure 3.26). Proportions were kept using a scale bar in FIpter and taking as a reference the total length of chromosome 1. Centromeric SATA signals were indicated on all the ideograms; interstitial SATA signals detected on chromosome 2, 3 and 4 were also indicated as shown in Figure 3.24. Another observation (also indicated in the same ideogram) made from PI stained chromosomes refers to a brighter p arm on one of the small autosomes (Figure 3.25), identified as chromosome 6

and also observed in the karyotype in Figure 3.23. Following Flpter measures from each chromosome, it was found sixteen subtelocentric and three submetacentric chromosomes as previously described on section 3.4.1.6.

At this point is important to mention that FISH with SATA demonstrated to be a useful resource for chromosome classification, improving accuracy of identification on mitotic spreads despite the small differences in size as observed with *O. karongae* when classifying chromosomes just by DAPI staining (see section 3.4.1.6). Thanks to the SATA results, the *O. karongae* karyotype from mitotic chromosomes was compared (DAPI and PI staining) and corrected to its present stage (Figure 3.20).

Moreover, interstitial SATA signals gave a clue about the kind of fusions chromosomes 2, 3 and 4 could represent. Chromosome 2 with an interstitial signal at about Flpter=0.90, suggests a telomere-telomere fusion (now subtelocentric), supported by the portion of the LG1 marker UNH995/UNH104 and with a possible q/p orientation (see Figure 3.19); chromosome 3 with an interstitial signal at about Flpter=0.68, suggests a centromere-telomere fusion (now subtelocentric); chromosome 4 with an interstitial signal about Flpter=0.46, suggests a centromere-telomere fusion (now submetacentric).

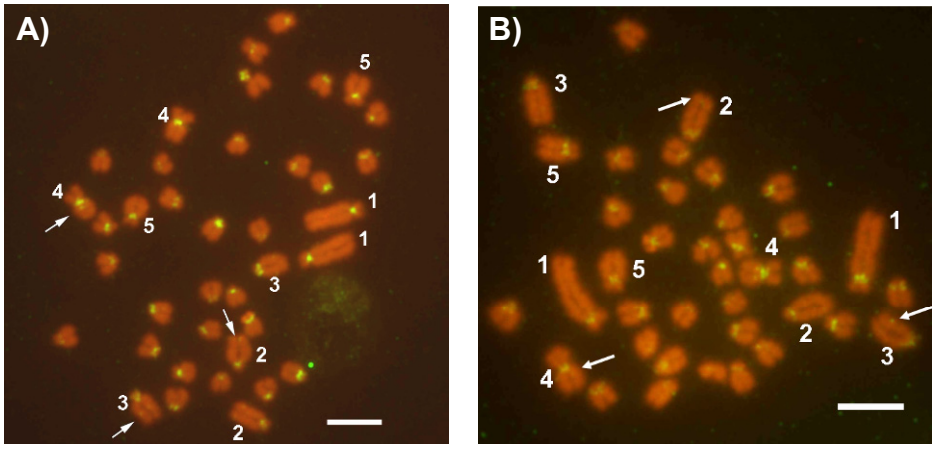

Figure 3.24 SATA hybridisations on male ZZ (A) and female WZ (B) mitotic chromosomes from *O. karongae*. SATA probes were labelled with Biotin-14-dATP and counterstained with propidium iodide. The first five larger chromosomes are indicated with numbers and interstitial SATA signals indicated with arrows on one of the homologous pairs. Picture A) represents the same chromosomes as Figure 3.23 displaying interstitial SATA signals on chromosome 2, 3 and 4. Scale bar = 5 µm.

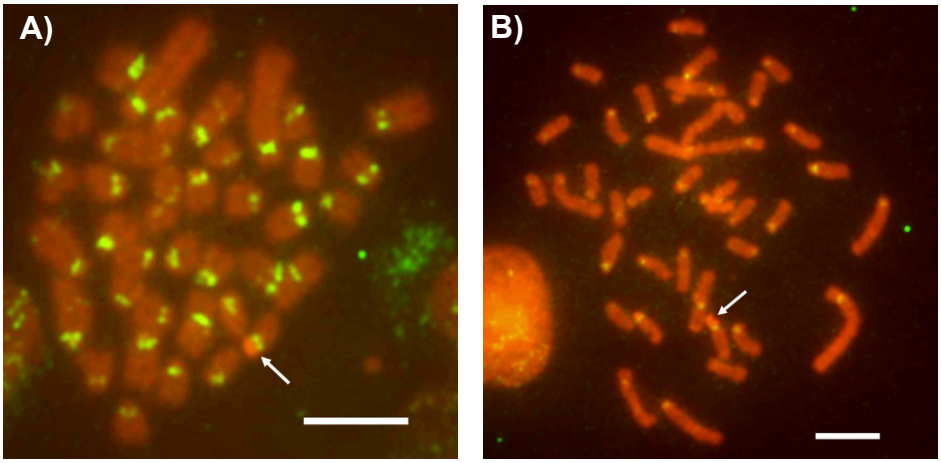

Figure 3.25 SATA hybridisations on male ZZ and female WZ mitotic chromosomes from *O. karongae*. SATA probes were labelled with Biotin-14-dATP and counterstained with propidium iodide. Arrows indicate a brighter p arm on one of the small chromosomes. Scale bar = 5 µm.

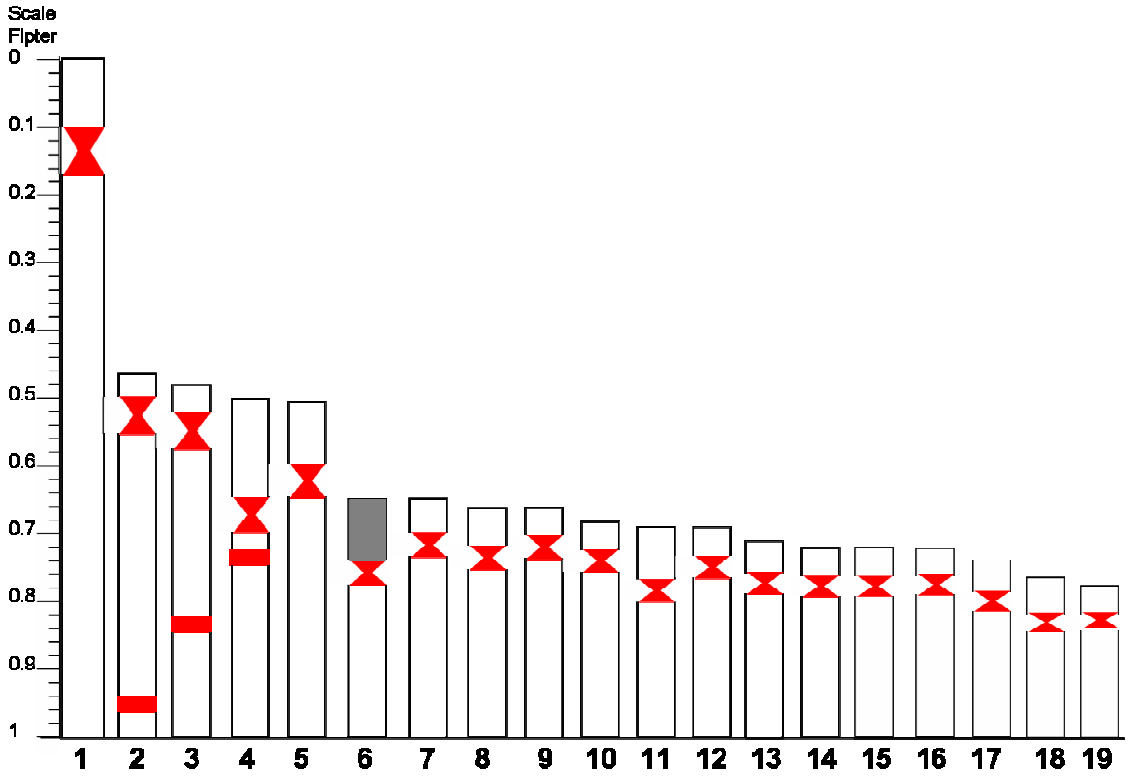

Figure 3.26 Physical mapping of SATA from mitotic spreads of *O. karongae.* Ideograms were classified according to total length (decreasing order) and centromere position (metacentric to telocentric) showing SATA hybridisations in red and brighter staining 6p arm under propidium iodide (PI) counterstain in grey. A scale bar in FIpter (chromosome 1) is displayed on the left side.

3.4.1.9 Physical mapping of repetitive telomeric sequence $(TTAGGG)_n$

To complement the work done towards the characterisation of chromosomes in tilapia, the conserved repetitive telomeric sequence (TTAGGG)n was physically mapped on the *O. niloticus* and *O. karongae* mitotic chromosomes. Hybridisation was observed at the telomeres of each chromosome for both species and there was no consistent evidence of interstitial signals for longer chromosomes even at low stringency (35% formamide). Figure 3.27 display some of the spreads from Nile tilapia that showed very weak signals at the middle of chromosome 1 as previously reported by Chew *et al.* (2002).

3.4.2 Meiotic Studies

3.4.2.1 Summary of FISH results from meiotic spreads

Meiotic studies were carried on at least 3 non-related *O. niloticus* males and 3 non-related *O. karongae*. Results obtained from this stage of the research were compared subsequently with mitotic studies to obtain a wide scope in relation to the genetic markers and centromere position along the chromosome and to construct the karyotypes. Moreover, late pairing regions were measured and integrated into the physical map of Nile tilapia. Two stages of late pairing on the large bivalent were recognised, early pachytene (maximum unpairing mean at Flpter= 0.80) and mid pachytene (minimum unpairing mean at Flpter= 0.93) with no stages observed in between (e.g. unpaired axial elements in central and subterminal regions as described by Carrasco *et al.* (1999).

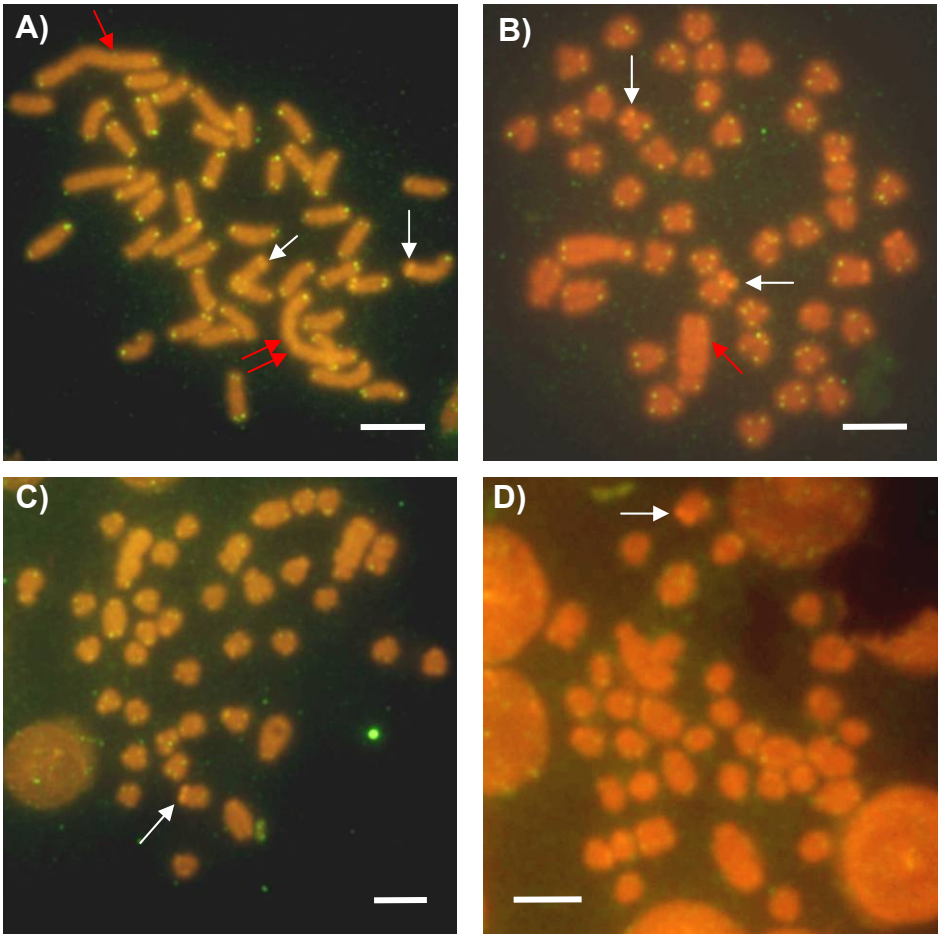

Figure 3.27 Physical mapping of the telomeric sequence $(TTAGGG)_n$ on mitotic spreads using low stringency (35% formamide). Telomeric probes were labelled with Biotin-14-dATP and counterstained with propidium iodide (PI). A) and B) Male XY *O. niloticus*; C) and D) Male ZZ *O. karongae*. White arrows indicate brighter p arms under PI staining. Red arrows indicate possible interstitial telomeric signals on chromosome 1. Scale bar = 5 µm.

Starting with the measure of the unpaired region in male XY Nile tilapia, an average unpaired region of 19.57% was observed in early pachytene, and an average unpairing region of 7.19% in mid pachytene, with respect to the total length of the larger bivalent (chromosome 1; Table 3.8). Centromeres were easily identified by DAPI stain on most of the spreads, observing them at the opposite end of the unpaired bivalent. This phenomenon was confirmed later when two markers from LG3 were detected by FISH on meiotic stages; marker *OniY*227 (227C) showed FIpter= 0.65, close to the maximum extent of the

unpaired region, and the gene dmrt4 (dmrt4C) showed Flpter= 0.22, close to the centromere that was measured at Flpter= 0.11 (Table 3.9). When comparing relative distances with mitotic spreads, Flpter of 0.57, 0.25 and 0.14 were observed for *OniY*227, dmrt4 and the centromere respectively (Table 3.10); the Flpter measurement ranges for each location were higher in mitotic spreads, indicating less accuracy when comparing with meiotic spreads.

Due to the better resolution from pachytene stage and the suggestion from several authors (Cheng *et al.,* 2001; Cunado *et al.,* 2000; Lynn *et al.,* 2002; Oliver-Bonet *et al.,* 2003; Peterson *et al.,* 1999) confirming that this stage is a good representation of LG distances and distribution, meiotic Flpter measures from SCs were converted to cM for maximum unpairing, *OniY*227, dmrt4 and centromere positions. Table 3.11 summarises these conversions.

Further comparisons were made in relation to LG3 in *O. niloticus* and a single mapping of one marker on an SC spread in *O. karongae*. Additionally, karyotypes with a superior resolving power (pachytene chromosomes in comparison to mitotic chromosomes) from *O. niloticus* and *O. karongae* are also displayed later on.

Table 3.8 Two observed pairing stages on the largest bivalent from SC spreads on pachytene meiotic cells in male XY *O. niloticus*.

UNPAIRING ORIGIN	N	Flpter Mean	% Unpaired	Range of variation
Maximum unpairing	2	0.80	19.57%	0.046
Minimum unpairing	3	0.93	7.19%	0.017

Table 3.9 Positional statistics calculated for two markers, *OniY*227 (227C) and dmrt4 (dmrt4C) that belong to LG3 (male XY *O. niloticus*). Data based on FIpter measurements from SC spreads at pachytene stage.

CATEGORY	FIpter *OniY*227	FIpter dmrt4	FIpter Centromere
N	17	11	17
Mean	0.65	0.22	0.11
Maximum	0.72	0.25	0.13
Minimum	0.60	0.18	0.09
Range	0.12	0.07	0.05
± SD	0.03	0.02	0.01

Table 3.10 Positional statistics calculated for two markers *OniY*227 (227C) and dmrt4 (dmrt4C) that belong to LG3 (male XY *O. niloticus*). Data based on FIpter measurements from mitotic spreads at metaphase stage.

CATEGORY	FIpter *OniY*227	FIpter dmrt4	FIpter Centromere
N	20	13	30
Mean	0.57	0.30	0.14
Maximum	0.65	0.36	0.24
Minimum	0.51	0.19	0.06
Range	0.14	0.18	0.17
± SD	0.04	0.05	0.04

Table 3.11 FIpter conversion to centimorgans (cM) from minimum and maximum unpairing, *OniY*227 (227C), dmrt4 (dmrt4C) and centromere location (male XY *O. niloticus*), measured from SC meiotic spreads during pachytene stage.

POSITIONS	N	Conversion SC FIpter to cM
Minimum unpairing	3	7
Maximum unpairing	2	19
***OniY*227**	17	33
dmrt4	11	74
Centromere	17	85

3.4.2.2 *O. niloticus*; orientation of unpaired region on chromosome 1

The chromosome 1 late pairing region was orientated with respect to the centromere by hybridising two markers and localising the centromere position by DAPI staining as mentioned in the previous section. When comparing meiotic and mitotic results on chromosome 1, it was found that one anchored markers (dmrt4) and the centromere showed very slight differences in the relative distances. *OniY*227, however, moved towards the maximum unpaired region (0.81) on the meiotic chromosomes with respect to the position on mitotic chromosomes, indicating a considerable change of the chromosome's internal distribution in the distal section of the q arm. The unpaired region and marker hybridisations are illustrated in Figure 3.28 and 3.29, and an ideogram comparison between meiotic and mitotic physical maps displayed in Figure 3.30.

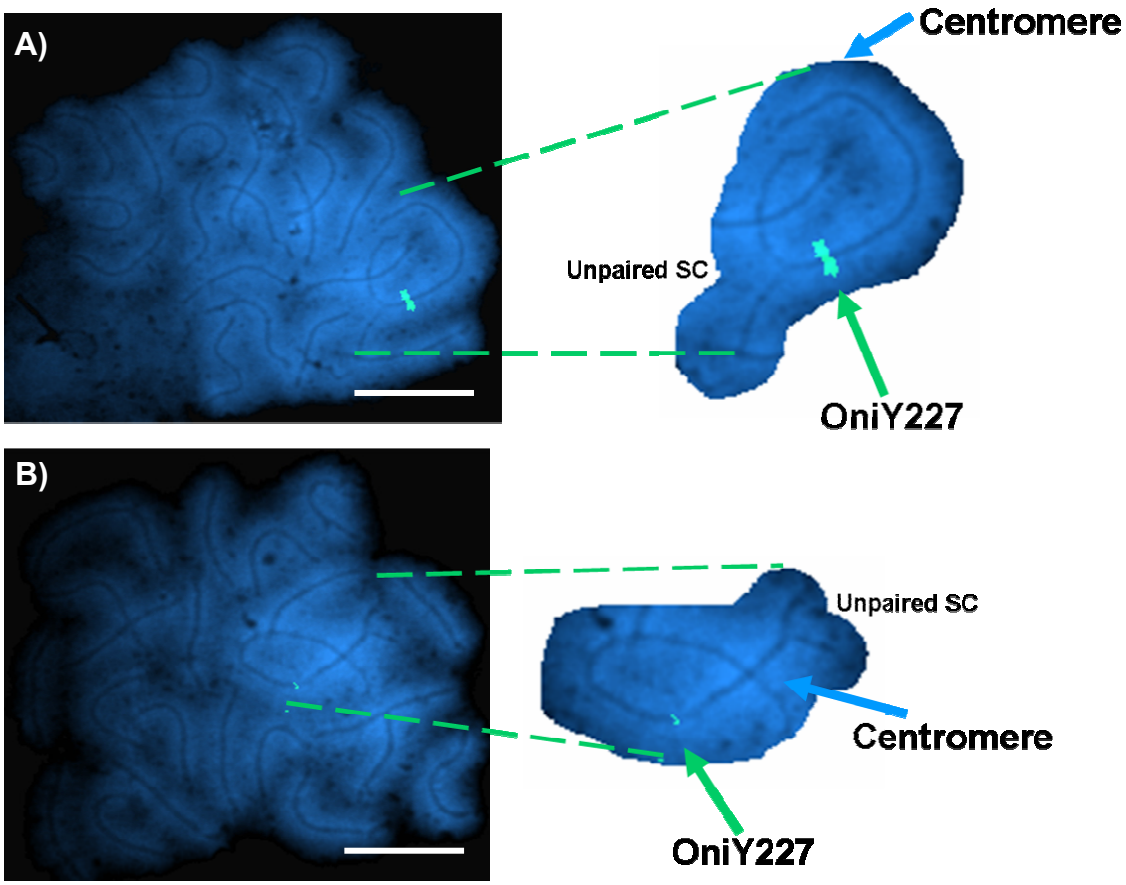

Figure 3.28 Male XY *O. niloticus* SC meiotic spreads showing a maximum (A) and minimum (B) unpaired region, also observed with more clarity by the enlarged bivalents on the right side. Marker *OniY*227 was hybridised by FISH (green colour from fluorochrome FITC) and chromosomes counterstained by DAPI. Scale bar = 5 µm.

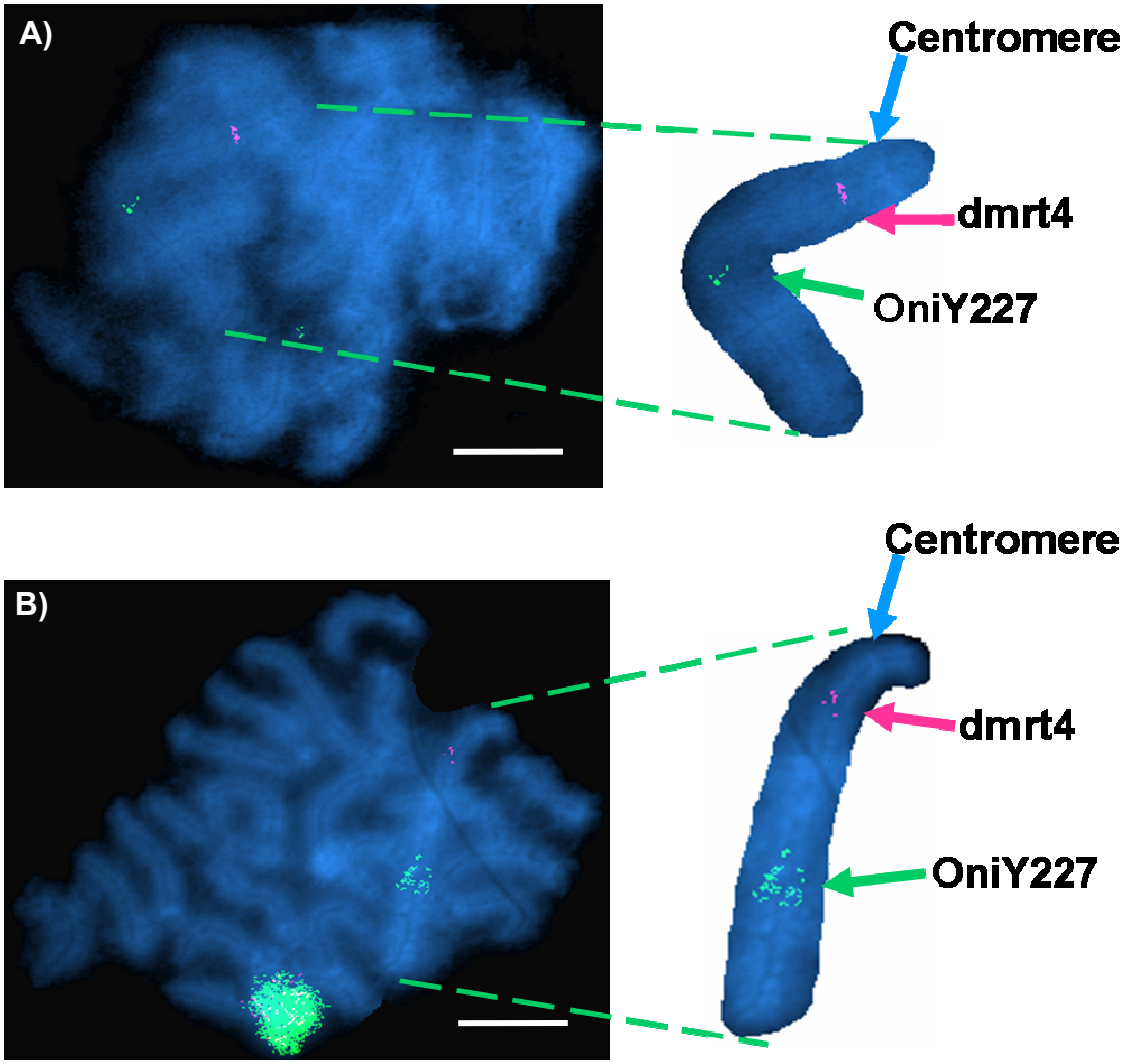

Figure 3.29 Male XY *O. niloticus* SC meiotic spreads showing in A) and B) the centromere position and dual hybridisation by FISH from two LG3 markers, *OniY*227 in green colour fluorochrome FITC) and dmrt4 in red colour (fluorochrome Cy3), also observed with more clarity by the enlarged bivalents on the right side. Chromosomes were counterstained by DAPI. Scale bar = 5 µm.

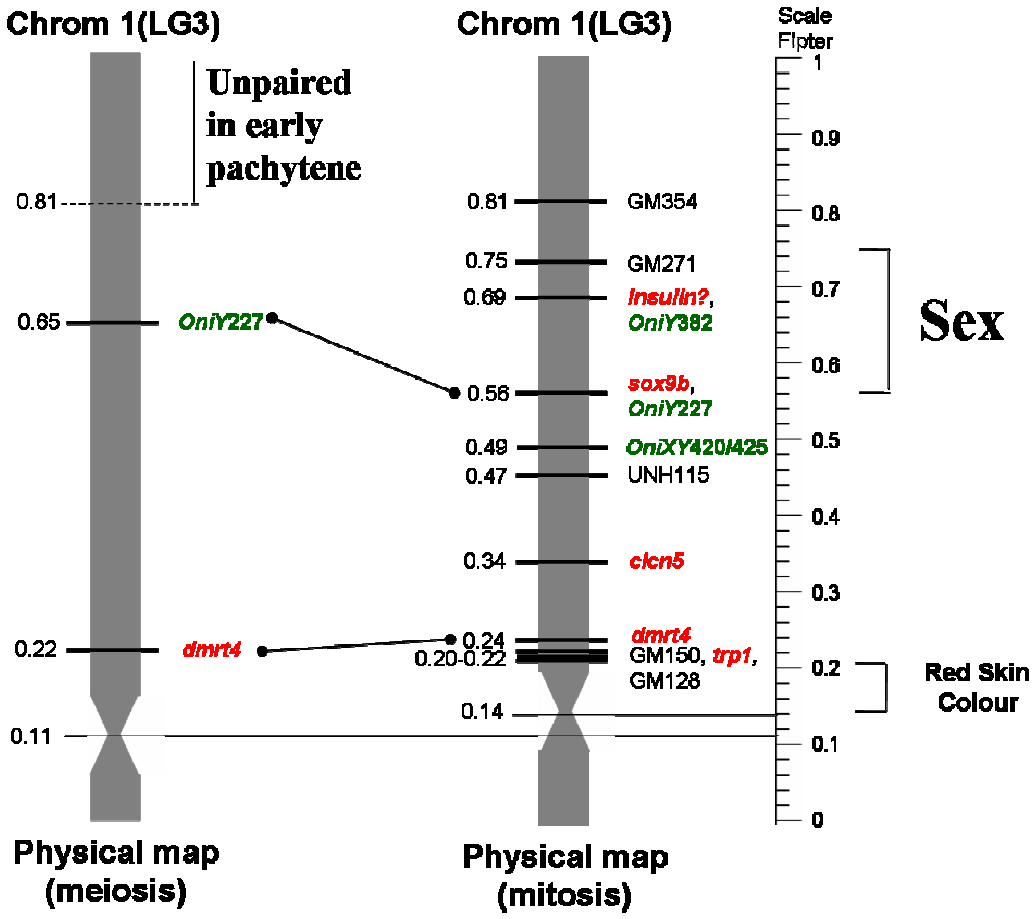

Figure 3.30 Ideogram comparison of meiotic (left) and mitotic (right) physical maps of chromosome 1 in *O. niloticus*, observing with connecting lines the anchor of two markers, centromere location and the observed maximum unpaired region during early pachytene. Putative sex determining locus and red skin colour locus are also indicated as references from previous observations. Scale ruler displayed in Flpter at the far right.

3.4.2.3 *O.niloticus*; a comparison of genetic linkage group 3 with SC physical map

When comparing the meiotic physical map with LG3 developed by Lee *et al.* (2005), the only available anchored marker was dmrt4 (dmrt4C) and an approximate position for *OniY*227 (Figure 3.31). For the conversion of relative distances (Flpter) to cM, LG3 total linkage map length was taken into account (95 cM). It was observed that the proportion of the total length in Flpter did not correspond to the total length in cM. Nevertheless, considering that GM354 (0

cM) doesn't hybridise at the end of the q arm of chromosome 1 in the physical map on mitotic spreads and the spatial differences observed for the anchored dmrt4 (74 cM from conversion of the physical map against 54 cM from the linkage map LG3), it was roughly estimated that about 20 cM (or 20%) at the top of the LG3 is still missing. Including this 20 cM into the linkage map, leads to a better match of marker distribution on meiotic spreads.

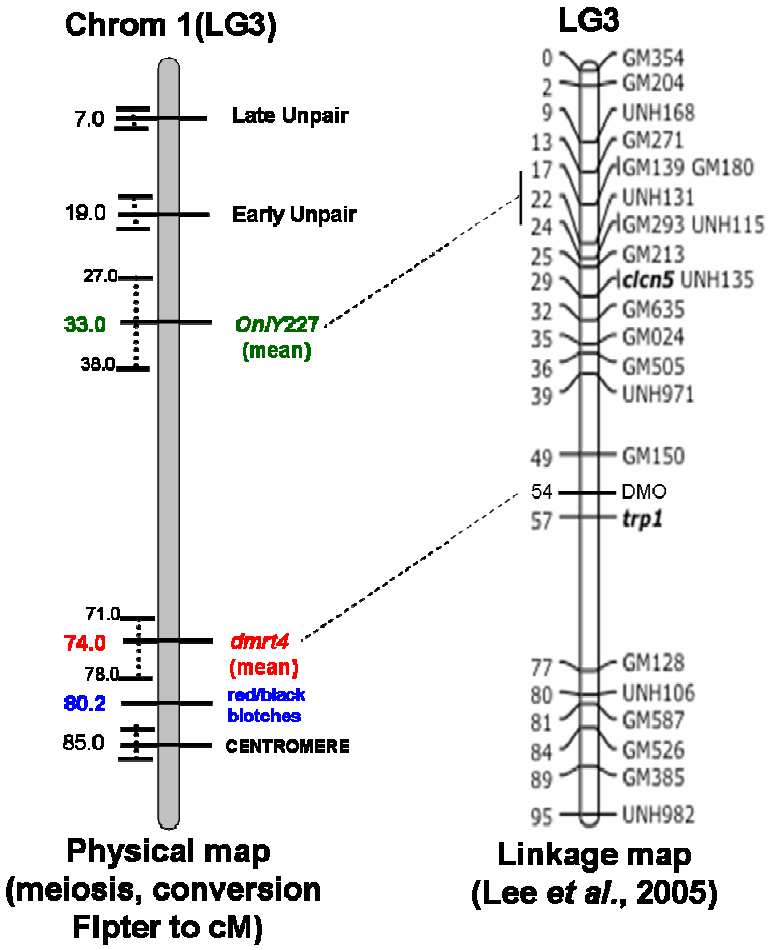

Figure 3.31 Ideogram from chromosome 1 and LG3 (developed by Lee *et al.,* 2005) comparison after conversion of FIpter to cM. Early and late unpaired region during pachytene, sex-linked marker (*OniY*227), gene dmrt4 (DMO) and the centromere are indicated on the ideogram (left) also displaying ranges of measures. *OniY*227 is suggested to be between GM271 and UNH115 markers according to previous mitotic FIpter observations whilst dmrt4 (DMO) gene is already anchored on LG3; both marker positions are indicated by dot lines. Red/black blotches or red skin colour locus is indicated in blue (according to Karayucel *et al.,* 2004).

3.4.2.4 *O. karongae*; hybridisation of the marker *OniY*227 on SC spread

The marker *OniY*227 in male ZZ *O. karongae* was physically localised along the larger bivalent during the pachytene stage as in *O. niloticus*. FIpter measures indicated that the localisation of this specific marker is well conserved even during pachytene stages when compared to *O. niloticus*. In *O. karongae* the centromere had an FIpter= 0.14 and the marker *OniY*227 an FIpter= 0.61 (Figure 3.32) whilst in *O. niloticus,* the centromere was at FIpter= 0.14 and the marker *OniY*227 at FIpter= 0.56. As was expected in a male ZZ fish, there was not unpairing observed in this long bivalent.

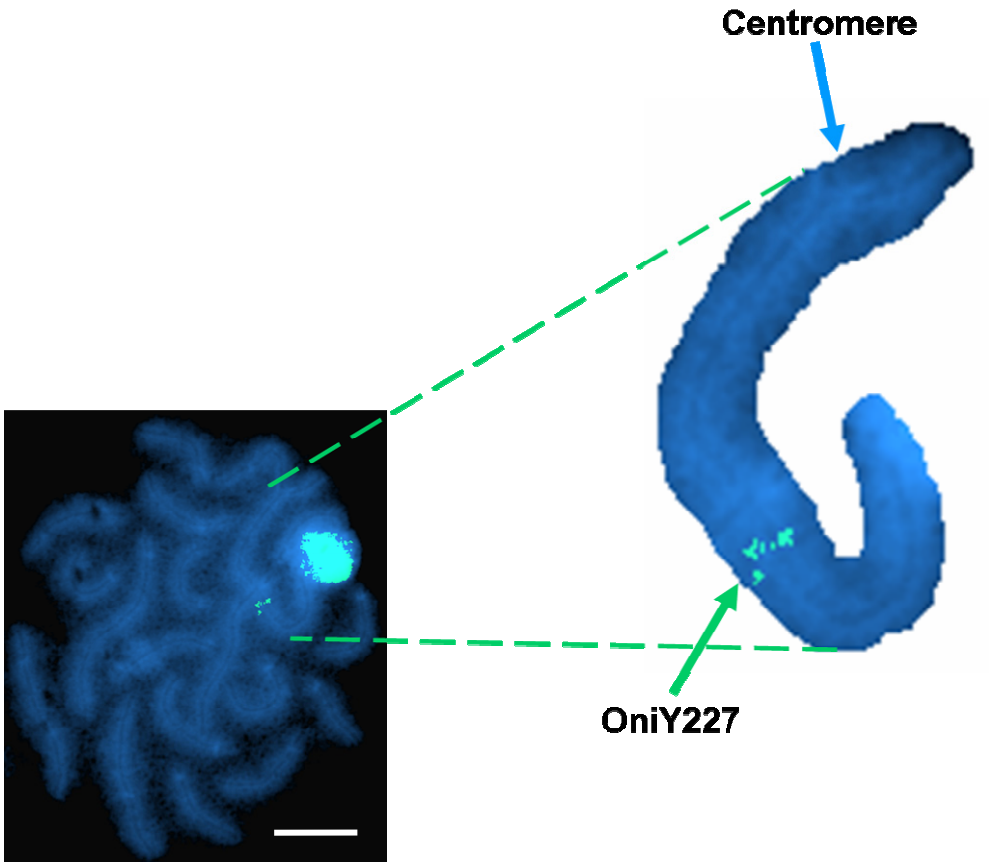

Figure 3.32 FISH on pachytene SC spreads from a male ZZ *O. karongae* meiotic cell showing the marker *OniY*227 (227C) on the larger bivalent at FIpter=0.61 in green colour (FITC fluorochrome). Chromosomes visualised by DAPI counterstain. Scale bar = 5 µm.

3.4.2.5 *O. niloticus*; karyotype based on meiotic SC spreads

An accurate meiotic karyotype was developed based on SC spreads. The three best spreads (bivalents fully paired and well dispersed) was made from three different individuals (one each). These three images were needed to obtain mean Flpter measurements from the total length and centromere position of each pair of bivalents. Using DAPI staining images turned into black and white images to obtain better contrast, n= 22 chromosomes (bivalents) were aligned firstly in decreasing order according to their total length and secondly from metacentric to telocentric chromosomes when chromosomes showed very similar length. The centromere was recognised by a brighter horizontal band when stained with DAPI (and a darker band in black and white images).

Once the chromosomes were sorted according to the above characteristics, they were straightened to compose the final karyotype. Three submetacentric (chromosomes 5, 7 and 11), sixteen subtelocentric (chromosomes 2, 3, 4, 6, 8, 9, 10, 13, 14, 15, 16, 17, 18, 19, 20 and 22) and three acrocentric/subtelocentric (chromosomes 1, 12 and 21) chromosomes were identified (see Figure 3.33). No metacentric or telocentric chromosomes were observed, agreeing with the results obtained from mitotic spreads. As a final note, an interstitial gap (absence of stain) was observed in several chromosomes (such as chromosome 2, 5, 10, 11, 21) followed by a strong stain (DAPI band after or before the gap); but only two of these chromosomes were consistently observed in the three meiotic spreads analysed, chromosome 2 (the second largest chromosome) at the middle of the q arm and chromosome 11 (the smallest submetacentric) just before the centromere on the p arm.

Finally, any other evident DAPI marks (secondary diffuse DAPI marks) were recorded (see Appendix X for measurements details).

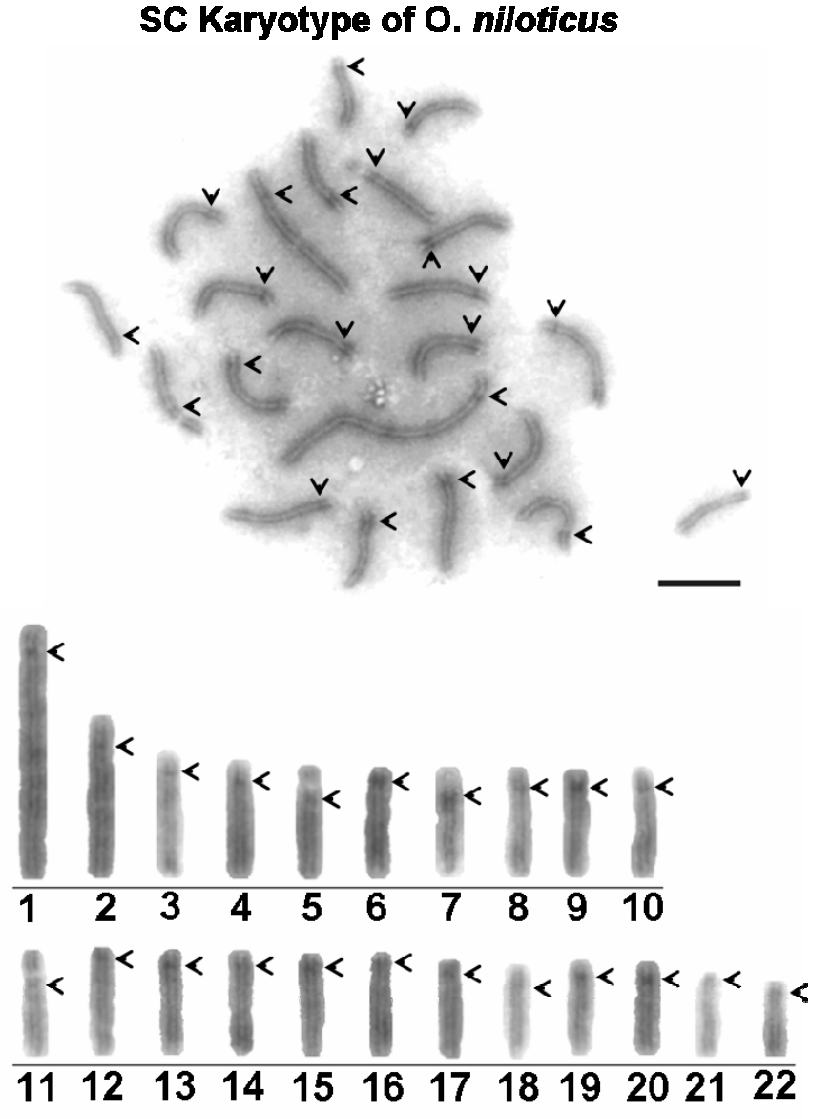

Figure 3.33 Male XY SC karyotype from *O. niloticus* showing the original spread (top) and the arranged chromosomes (bottom). Originally counterstained by DAPI and converted into a black and white image. Arrow heads indicate centromere positions. Scale bar = 5 µm.

3.4.2.6 *O. karongae*; karyotype based on meiotic SC spreads

A similar procedure was followed in *O. karongae* for the construction of an accurate meiotic karyotype based on SC spreads. A selection of the three best spreads was made from two different organisms to obtain mean FIpter measurements for the total length and centromere position of each bivalent. Using DAPI staining images converted into black and white to obtain better contrast, n=19 chromosomes were aligned firstly in decreasing order according to their total length and secondly from metacentric to telocentric chromosomes when chromosomes showed very similar length. The centromere was recognised by a brighter horizontal band when stained with DAPI (and a darker band in black and white images).

Finally, the chromosomes were sorted and straightened to compose the final karyotype. The results agree in general with data obtained from mitotic spreads stained with DAPI and PI (with SATA hybridisations). Meiotic DAPI stained results demonstrated three submetacentrics (chromosomes 4, 6 and 11), fifteen subtelocentrics (chromosomes 1, 2, 3, 5, 7, 8, 9, 10, 12, 13, 15, 16, 17, 18 and 19) and one acrocentric/subtelocentric (chromosome 14) (see Figure 3.34). No metacentric or telocentric chromosomes were observed. This analysis not only helped to construct the karyotype but also to obtain more specific classification through observations on interstitial bright bands and gaps not evident on mitotic spreads.

An interesting observation from the three spreads analysed was the evident gap (absence of stain) in chromosomes 5, 13, 15 and possibly in the middle of the submetacentric chromosomes 4 and 11. The gap followed by a brighter band on chromosome 5 was also easily observed in *O. niloticus*.

Chromosome 5 in *O. karongae* is at least partially homologous to chromosome 2 in *O. niloticus* demonstrated by FISH using the markers Wt1a, *OniXY*420/425? and brain aromatase from LG7 as mentioned in previous sections (mitotic studies). Both homologous chromosomes now demonstrate this gap. The presence of this morphology could suggest an ancient telomere-centromere fusion (although no signals from SATA or telomeric sequences were observed using FISH) or simply a simultaneous accumulation/deletion of GC elements in a contiguous area detected by DAPI stain as a bright and a dark band.

3.5 Discussion

3.5.1 Putative sex chromosomes and sex linkage groups

There are conflicting views and evidence regarding the localisation of the putative sex determination locus in *O. niloticus*. A range of data suggest that chromosome 1 is implicated such as an unpaired region on the largest chromosome (chromosome 1) during early pachytene (Carrasco *et al.,* 1999) only observed on SC spreads coming from a fish with XY genotype but not from an XX or YY; a great heterochromatin accumulation on the distal second half of the q arm on chromosome 1 demonstrated by the presence of transposable elements such as SINE, Ron-1, CiLINE2 sequences (Oliveira *et al.,* 2003); (Harvey *et al.,* 2003b); the differences observed on cross hybridisation using microdissected distal q arms from chromosome 1 as probes from XX and YY fish (Harvey *et al.,* 2002b) giving at the same time evidence of “sex chromosomes” in an early stage of differentiation; and the FISH localisation of

three SLAMS on the same distal area of chromosome 1 (Ezaz *et al*., 2004b). However there is equally convincing data to suggest that LG1 contains the main sex determination locus.

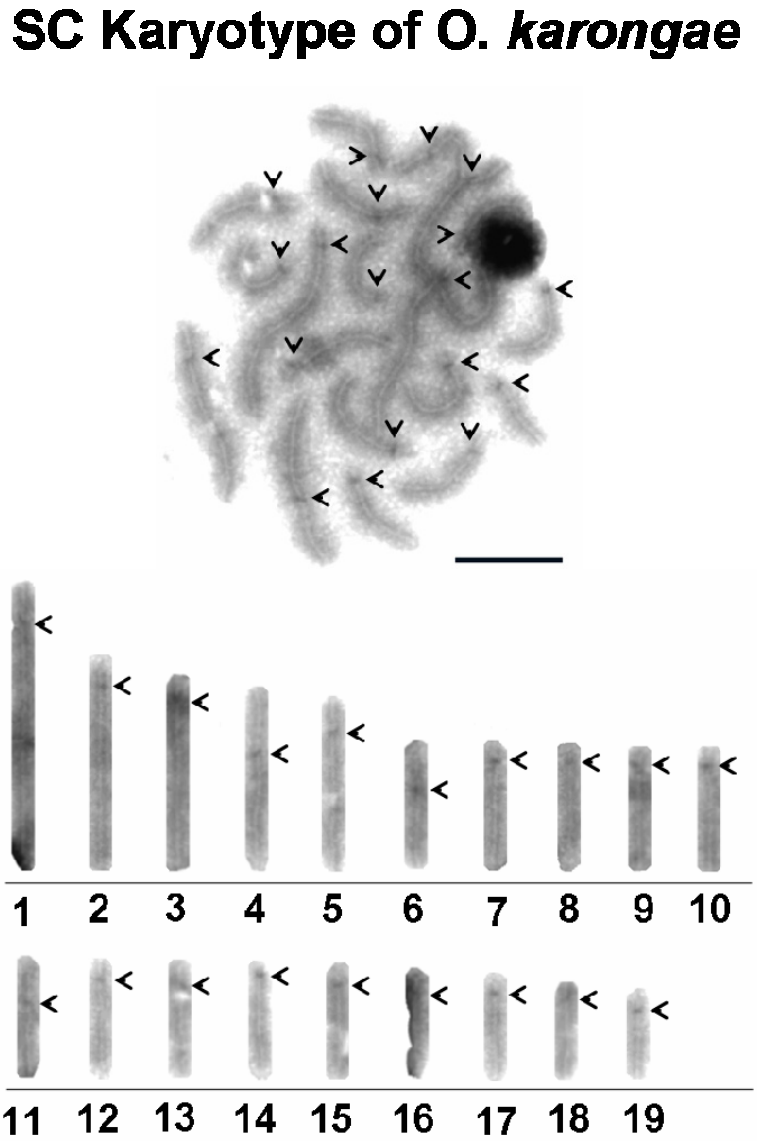

Figure 3.34 Male ZZ SC karyotype from *O. karongae* showing the original spread (top) and the arranged chromosomes (bottom). Originally counterstained by DAPI and converted into a black and white image. Arrow heads indicate centromere positions. Scale bar = 5 µm.

The evidences are the clear differences in recombination between the LG1 from males and females (Lee *et al.,* 2003). Moreover, in two families of Nile tilapia *O. niloticus,* the sex locus was mapped on LG1 at 34.9cM (between

the gene Wt1b, 32.4cM, and the marker UNH995, 38.3cM) (Lee and Kocher, 2007). Considering all these evidences, assignment of LG1 to chromosome 1 was expected.

Nevertheless, Cnaani's *et al* (2007) FISH observations and the present physical map in *O. niloticus* along with the other six tilapia species analysed in this study (with n=22 chromosomes; *O. aureus, O. mortimeri, O. mossambicus, Sarotherodon galilaeus, Tilapia rendalli, T. zillii*) demonstrate clearly that LG3 is the one that belongs to the larger subtelocentric chromosome (chromosome 1) and LG1 to a small submetacentric chromosome. *O. karongae* (n=19), having different number of chromosomes, also hybridise markers from LG3 onto chromosome 1 but with LG1 markers localising to chromosome 2.

Some of the explanations for these findings could relate to the evidence of an early stage of differentiation (Harvey *et al.,* 2002b), reflecting a very close transition between males and females supported by the observed influence of additional autosomal sex modifying loci (Hussain *et al.,* 1994; Mair *et al.,* 1991a) and environmental factors such as temperature and pH of water (Baroiller and D'cotta, 2001).

It is possible that a transition between ZW to XY or vice versa occurred during evolution, and in species with a combination of X, Y, W and Z chromosomes, could give rise to a different genotypic sex system, determining sex by a combination of sex determining and regulatory genes distributed in different sex chromosomes and autosomes (Ezaz *et al.,* 2006). A clear example of this transition could be illustrated with the epistatic interaction of the sex determining loci observed in *O. aureus* between LG1 (UNH104 alleles) and

LG3 (UNH131 alleles) and suppression of recombination when compared both males and females LGs (Lee *et al.,* 2004).

Another piece of research recently indicated UNH168 (LG3) as one of the possible candidate marker related with sex determination in *Oreochromis* spp. (Shirak *et al.,* 2006). The physical distribution of markers along the q arm on chromosome 1 also demonstrates an evident suppression of recombination close to the putative sex determination locus (UNH168) between the marker GM354 and the gene CLCn5 where the three SLAMS (*OniXY*420/425, *OniY*227 and *OniY*382) were also mapped.

This convergence of information (present physical map and previous linkage analysis) at this stage suggests then two epistatic sex determining loci in LG1 and LG3 with stronger influence on phenotype from LG1 in *O. niloticus* and LG3 in *O. aureus* where an alternate master gene could lie. Because the present chapter is mainly focused on physical mapping from chromosome 1, only further discussion and observations about the suppression in recombination region (a putative sex determining locus) on LG3 is extended below.

3.5.2 Construction and integration of the physical map from Chromosome 1 and other chromosomes

Probes prepared by DOP-PCR often showed hybridisations with low reproducibility and high level of background. On the other hand, probes prepared by nick translation showed hybridisations with higher reproducibility and lower level of background, which implies more specificity. As a result most

of the probes were labelled by nick translation for the construction of the physical map in *O. niloticus*, *O. karongae* and the other six species. The nick translation technique has been demonstrated to be the best and most common option for probe labelling in different plants (Cheng *et al.,* 2001; Peterson *et al.,* 1999) and animals (Chew *et al.,* 2002; Phillips *et al.,* 2006a; Yeh *et al.,* 1996) using different probe sources such as plasmids or PCR amplifications.

It is also relevant to mention that spatial arrangements from BACs containing different linkage groups were observed in several occasions in interphase cells by dual hybridisations using nick translation techniques (Figure 3.16, (A) and (G)), confirming the segregation of markers belonging to different LGs in a spatial distance. Also, hybridisation on interphase cells frequently serves as positive controls when looking for specific signals after FISH (Iturra *et al.,* 1998).

Some BACs displayed a range of sub-optimal signals. These included 1) diffuse signals, 2) multiple signals on the same chromosome, 3) multiple signals on different chromosomes, 4) a very weak signal or 5) no signal at all. BAC clones containing GM271 (LG3), Sox9b and *OniXY*420/425 were the model cases in that secondary or multiple signals were present and even more, showing more affinity (stronger signal) to a different location on chromosome 1 (GM271) or to another chromosome (Sox9b and *OniXY*420/425) as well as weaker signal at the expected locus or chromosome. It could be the case that repeat sequences are common to both locations but with more copies on the unexpected location or the possibility that the BAC clone may have an asymmetric chimera, having mostly chromosome 2 sequences and a small

piece from chromosome 1 as suggested by Ezaz *et al.* (2004b) for the BAC clone containing *OniXY*420/425.

The hybridisation of the 420/425C BAC clone on chromosome 1 was supported by the linkage map developed by Ezaz *et al.* (2004b), demonstrating a close association of the three SLAMs studied, to the sex determining locus. *OniXY*420/425, *OniY*227and *OniY*382, had recombination distances of 20 cM, 17 cM and 13 cM respectively from the sex determining locus in Nile tilapia. Despite *OniY382* sequence coexisting in the same BAC clone as the Insulin gene (InsulinC), the arrangement of the three AFLPs on chromosome 1 seems to agree not only with the results of Ezaz *et al.* (2004b) but also with the orientation and proximity of the unpairing region (Approx. 12% apart).

Despite the two insulin 1 probes hybridising to different locations using different BAC clones (InsulinC and 16T), the signal from each of these BACs was strong/medium and apparently specific either to chromosome 1 (LG3) or to a small chromosome (LG10). It is proposed then in further studies to hybridise both BAC clones in a dual FISH and confirm whether a secondary signal from InsulinC hybridise at the same location than 16T using a low threshold picture.

It is relevant to mention about the implications that heterochromatin accumulation on the long arm of chromosome 1 has for FISH techniques. The case of the two insulin BAC clones hybridising to two different locations after demonstrate to have the same sequence with no mutations (at least in the 3.2 Kb sequenced by the University of Dalhousy) is a clear example of possible FISH misinterpretations. Many of the BAC clones were appearing on the q arm of chromosome 1, mainly on the heterochromatin area when the signal

threshold was reduced; otherwise they appeared on a different location with a weak signal. The high amount of repeat sequences accumulated on this chromosome area, creates great affinity for many BAC clones despite the 50 fold blocking DNA used during the hybridisation step. The best alternative in that case is to combine physical and linkage map analyses as it was applied in the present research. There would be then necessary in the case of SLAMs and SOX9b to genotype-analyse them along with other markers from LG1 and LG3 to confirm locations on chromosomes.

So far, in *O. niloticus,* thirteen markers and genes were physically mapped on chromosome 1, three on LG7, two on LG1, two on LG6, two on LG10 and one on LG12. The only differentiated chromosomes with assigned LGs were chromosome 1 with LG3 and chromosome 2 with LG7, although LG1, LG6, LG10 and LG12 were confirmed to be on different small submetacentric or subtelocentric chromosomes by dual hybridisation. The small chromosome with LG1 is longer (34% relative to chromosome 1) than the other 3 assigned chromosomes (24-28% relative to chromosome 1). Similar methodologies had been applied in rainbow trout and zebrafish for the assignment of linkage groups to specific chromosomes (Phillips *et al.,* 2006a; 2006b) using a combination of factors, e.g. BAC probes, relative sizes, chromosome arm ratios and centromere probes. Following the previous example, to complete the full assignment of linkage groups to the small Nile tilapia chromosomes, it would be necessary to build a new *O. niloticus* mitotic karyotype based on two BAC probes hybridised from each linkage group and total relative chromosome sizes with respect to chromosome 1 calculated in FIpter and finally hybridising SINE sequences to improve the identification of homologous pairs and ordering

process. Then, it should be possible to assign with great accuracy every LG to the small chromosomes arranged on the mitotic karyotype. Full assignment of the 23 LGs onto the whole karyotype of *O. niloticus* would be of great use for further studies, such as to identify any differences in the other six tilapia species studied (*O. aureus, O. mossambicus, O. mortimeri, S. galilaeus, T. rendalli, T. zillii*) even though they showed well conserved LG distribution for LG1 and LG3.

In contrast to *O. niloticus*, where only mitotic spreads were used to assign linkage groups, both mitotic and meiotic spread were exploited to assign LGs in *O. karongae*. Linkage groups 1, 3 and 7 were then assigned to chromosomes 2, 1 and 5 respectively in this species. The remaining chromosomes were characterised in an ideogram (Figure 3.26). Meiotic spreads gave greater resolution for karyotyping purposes and still resembled the somatic karyotype as was observed in zebrafish (Wallace and Wallace, 2003) when comparing mitotic and meiotic karyotypes.

In *O. niloticus*, there seems to be poor correspondence between the length of the chromosome and the size of the linkage group at the stage of the present genetic linkage map presented by Lee *et al.* (2005). Chromosome 1 has an LG size of 95 cM and chromosome 2 of 65 cM (the largest chromosomes) whilst within the remaining linkage groups that must be assigned to small chromosomes, some LGs have 94 cM (LG16 + LG21) (Shirak *et al.,* 2006), 77 cM (LG4) or 65 cM (LG12). Phillips *et al.* (2006a) suggests that the genetic recombination in zebrafish is elevated in some of the smaller chromosomes (subtelocentric or acrocentric) in order to ensure one cross-over per chromosome arm. Also it is valid to consider that LG3 is shorter than

expected due to suppression of recombination or possibly LG3 is still incomplete and requires further anchoring of new markers. According to the present physical map (Figure 3.11), it is evident that LG3 is not covering the whole chromosome at least on the q arm due to a gap observed between the physical position of marker GM354 (Flpter= 0.81, 0 cM) and the telomeres.

The suspected suppression of recombination in sex chromosomes is supported by the contraction-expansion areas observed along the LG3 when comparing with the novel physical map. The contraction represents suppression in recombination and the expansion an increment in recombination activity. In rainbow trout (Phillips *et al.,* 2006b) observed that male recombination is greatly suppressed near the centromere but inflated near the telomeres, but in the case of *O. niloticus,* chromosome 1 showed the opposite, with greater recombination near the centromere and suppressed recombination towards the telomeres (from GM128 to Clcn5, the rate is 3.43 cM = 1 Flpter and from Clcn5 to GM354, 1 cM = 1.62 Flpter). Changes in the expected recombination pattern are often associated with the presence of heterochromatin, which appears to be limiting recombination events on the distal part of the long arm (1q) on chromosome 1.

With the sole exception of *O. karongae*, LG1 mapped to a small (undefined) chromosome). For *O. karongae* (with its distinct karyotype 2n=38) LG1 clearly mapped to a large chromosome (chromosome 2). However LG3 and LG7 in *O. karongae* appeared to map to the same position as the other tilapia species studied onto chromosome 1 and 5 (same than chromosome 2 in the other species) respectively. This demonstrates that there is in general a well conserved chromosome composition among these species with different

chromosome number. Further discussion in relation to the karyotype comparison of these two species is given in section 3.5.4.

There is no question about the requirement for completing a study with the complete assignment of all LGs onto the chromosomes and the benefits that it implies for a broad understanding of the genome and its dynamics in tilapia. Nevertheless, at this stage is possible to confirm that the physical map developed in this study represents a solid base from where further markers and genes could be anchored and moreover, perform more accurate comparisons and detect changes in the distribution and localisation of genes of interest.

3.5.3 Comparison with previous physical maps from Chromosome 1

Previous studies on physical mapping in Nile tilapia (Boonphakdee, 2005; Cnaani *et al.,* In review; Ezaz *et al.,* 2004b; Harvey *et al.,* 2003b; Martins *et al.,* 2004), represented mostly physical approximations due to signal dispersion (DOP-PCR technology) signals or because of the lack of relative measurements. Only general comparisons were then possible during the anchoring of new markers.

Boonphakdee (Boonphakdee, 2005) identified *OniX*Y420/425, *OniY*227, *OniY*382, dmrt1,2,3 and insulin at the same position in chromosome 1 with Flpter = 0.60 and Sox9b at Flpter = 0.70, but the signals obtained were not precise (see section 3.5.2), and in some cases (dmrt1,2,3, insulin and Sox9b), there are now doubts about the location of the BAC clones identified on chromosome 1. Nevertheless, this first physical map of chromosome 1 gave a wider insight about the possible location of the master sex determination gene

or at least one of the main loci involved in sex determination. As mentioned in section 3.5.1, previous evidence support the idea of the presence of a sex determining locus on chromosome 1, such as the high concentration of transposable elements and other repetitive DNA sequences on the distal half of the q arm (Harvey *et al.,* 2003b; Oliveira *et al.,* 2003), and sequence differences found between the larger chromosome pair (Harvey *et al.*, 2002b), and unpairing region of the larger bivalent in early pachytene exclusively in XY fish (Carrasco *et al.,* 1999).

The present physical map on chromosome 1 was compared with the physical map presented by Cnaani *et al.* (2007). Differences between the distances, but not in the order of the markers (between GM354-GM271 and UNH115-Clcn5) were found. These differences could be related to specific arrangements among strains, individuals or phenotypic sexes as demonstrated in females and males with the same genotype in Blue tilapia (Campos-Ramos, 2002), Zebrafish (Wallace and Wallace, 2003), mice (Lynn *et al.,* 2005) and crested newts (Wallace *et al.,* 1997), although there are only approximate positions given for the markers mapped by Cnaani *et al.* (2007) that could lead to plain technical differecnes.

Another possible consideration to explain the differences observed in marker distribution on physical maps (if not related to differences in genome distribution) is the chromosome's preparation technique and hybridisation conditions. During chromosome preparation, hypotonic shock and fixation steps vary in how well the chromosomes spread and the clarity of their morphology (Henegariu *et al.,* 2001). In addition, during hybridisation, the use of blocking

DNA at the right concentration, have been demonstrated to be crucial to avoid false signals or weak positive signals. Nevertheless, a comparison of several spreads (running dual hybridisation and generating average measurements with SD± for every marker, see Appendix VII) was done to give the required confidence to position the markers on the ideograms developed in this study. In this way, it is less likely to consider chromosome preparation and hybridisation issues as the main reasons for the physical differences observed between Cnaani's version (Cnaani *et al.,* 2007) and the present version.

Sex-determination regions are often associated in higher vertebrates with heterochromatic regions, which are commonly rich in G/C sequences (Silva and Yonenaga-Yassuda, 1998). Accumulation of heterochromatin is often the first visible sign of sex chromosome differentiation in fish too, as observed in the Neotropic electric eel with an XX:XY sex chromosome system (De Almeida-Toledo *et al.,* 2001b). The end of the q arm in chromosome 1 is characterised for having a major accumulation of heterochromatin as shown by Harvey *et al.* (2003b) and Oliveira *et al.* (2003) who found concentrations of several SINE repetitive sequences there. In addition, CiLINE2 is uniquely enriched along the terminal two-thirds of the long arm of the same chromosome (Oliveira *et al.,* 1999). This is probably the reason why many BAC clones have great affinity to hybridise in this area. Current findings also demonstrate an association between the beginning of the heterochromatin region (Flpter= 0.70 - 0.80, see Figure 3.36) on chromosome 1 and the measured origin of the unpaired region on meiotic spreads (Flper= 0.80, see Table 3.8). Going further, a cM comparison between the meiotic physical map and the LG3 map revealed that the early unpaired origin is located at 0 cM where the marker GM354 is

genetically mapped, supporting the origin of the heterochromatin concentration at this point. This comparison also demonstrate that approximately 20 cM (20%) at the top of LG3 is still absent in this linkage group, and may correspond to the unpaired region (Figure 3.31).

Further strong evidence from this study that supports the idea of a sex-determining locus close to the origin of the LG map was the observation of a contracted zone in the area between the markers clcn5 (Flpter=0.34) and GM354 (Flpter= 0.81), and more specifically between UNH115 (Flpter= 0.47) and GM354 (Flpter= 0.75) where the three SLAMs were located together with Sox9b and insulin genes. This observation is supported also by the physical map from Cnaani *et al.* (In review). Nevertheless, it was found in normal crosses, by linkage mapping, that the closer SLAM *(OniY382)* is still about 13 cM distant from the sex-determining spot (Ezaz *et al.,* 2004b). Shirak *et al.* (2006) suggested that marker UNH168 in *O. aureus* × *O. niloticus* hybrids may be closely associated with one of the sex determination loci. Lee *et al.* (2004), studying males and females from *O. aureus,* also found a strong suppression of recombination in ZZ males in LG3 between the markers GM354 and UNH131. Combining previous physical and genetic maps findings on chromosome 1, it is suggested that a sex-determination gene (if not the main one) must be situated in the area between the marker GM271 (Flpter= 0.75, 13 cM) and GM354 (Flpter=0.81, 0 cM) which represents 6% or 13 cM maximum gap.

Fish models such as the stickleback *Gasterosteus aculeatus* demonstrates that a sudden suppression in recombination observed between male and female linkage maps was associated with the sex-determining locus,

supported later by poor sequence homology in this region between the X and Y chromosome (Peichel *et al.,* 2004). In the case of *O. niloticus*, as mentioned before, sequence differences between the sex chromosomes were also inferred by differences in comparative hybridisation of X and Y chromosome-derived probes (Harvey *et al.,* 2002b). Despite the differences in sequence between sex-chromosomes, no morphological differences were shown between X and Y chromosomes in these two species (*G. aculeatus* and *O. niloticus*), suggesting a nascent Y chromosome in early stage of differentiation possibly with less than 10 million years old (Harvey *et al.,* 2002b).

It was suggested here that LG1 is located in a subtelocentric/submetacentric chromosome of *O. niloticus* representing about 34% of the total length of chromosome 1. From the ideogram of Martins *et al.* (2004), chromosomes 4, 5 and 7 could be the possible candidates. Chromosome 4 displayed SATB repeats on the p arm, chromosome 5, SATA repeats also on the p arm, and chromosome 7, SATA repeats on a half of the p arm (see Figure 3.35); Using the SATA/SATB probes together with the marker UNH995/UNH104 (LG1) could help to clarify the specific small chromosome pair holding LG1 in tilapia.

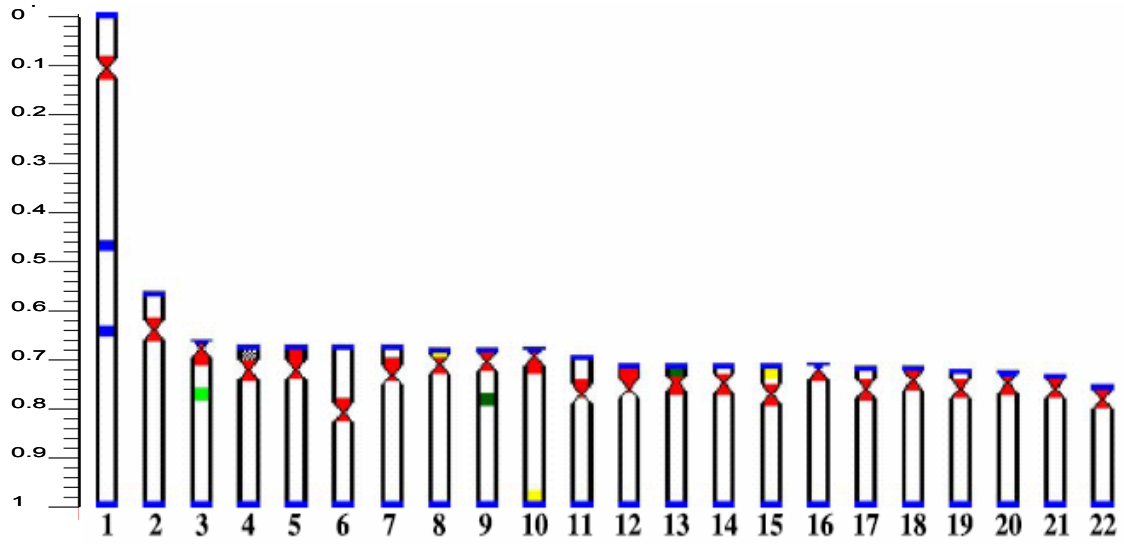

Figure 3.35 Ideogram representing the physical mapping of the repetitive sequences in the chromosomes of the Nile tilapia (*O. niloticus*). The chromosome position of the different sequences is distinguished by colour: red, SATA; speckled in black, SATB; yellow, 45S rDNA; blue, telomere; faint green, 5SrDNA type I; dark green, 5S rDNA type II. (Source: Martins *et al.,* 2004), with the addition of an FIpter scale bar on the left side).

3.5.4 Comparison of mitotic and meiotic physical maps

Meiotic and meiotic based maps can vary greatly. For example meiotic pachytene chromosomes are often more than 10 times longer than somatic metaphase chromosomes (Cheng *et al.,* 2001). In cichlids, the proportions or relative sizes of the small chromosomes vs chromosome 1 differ (Turner *et al.,* 2001a). On mitotic spreads of *O. niloticus*, chromosome 2 is about 50% of the total length of chromosome 1, whilst on meiotic spreads chromosome 2 represents 70% of the total length of chromosome 1. The same phenomenon is observed in *O. karongae* when comparing the four medium sized chromosomes against chromosome 1. These differences between somatic and germinal chromosomes could be explained due to the different rate of chromosome contraction (DNA-histones-nuclear matrix packing) through the process of meiogenesis or mitogenesis (Turner *et al.,* 2001b). It is also explained by a

delayed synapsis or synapsis anomalies involving heterochromatin regions (Codina-Pascual *et al.,* 2006) affecting the relative proportions of medium and small chromosomes in relation to chromosome 1 when comparing mitotic and meiotic spreads. But not only the proportion of the chromosome sizes changes, there are also differences in the relative distances of the hybridisation signals when comparing Flpter measurements in meiotic and mitotic stages (Boonphakdee, 2005). For instance, on chromosome 1, Boonphakdee obtained with the AFLP marker *OniY*227 an Flpter= 0.57 in mitotic spreads and Flpter= 0.64 in meiotic spreads; and for the gene DMO (dmrt4) an Flpter= 0.18 in mitotic spreads and Flpter= 0.22 on meiotic spreads. Ezaz *et al.* (Ezaz *et al.,* 2004b) also point to the differences observed between measurements made in mitotic and meiotic stages from the physically mapped AFLPs.

Another factor that probably accounts for some of the variation in the relative distances within a chromosome is that telomere sequences are longer in germline tissue than in somatic tissue as observed in humans (de Lange *et al.,* 1990) and in *O. niloticus* (Chew *et al.,* 2002). Nevertheless, telomere variations could be hard to detect under FISH techniques.

Finally, the present study showed that pachytene stages facilitated a more precise measurement of relative distances (Flpter) from total lengths, centromeres and BAC probes in comparison with mitotic stages. The main evident reason is that at late mitotic metaphase stage (when chromosomes are fully condensed; (Czepulkowski, 2001), a loss in the sharpness of the general chromosome structures and a reduction of the length differences among the chromosomes, made relative measurements and comparisons more prone to

errors. It is suggested then that FISH on SC spreads should improve resolution during physical localisation of the two putative sex determining loci (in relation to LG1 and LG3).

3.5.5 Karyotypes from *O. niloticus* and *O. karongae*

Because of the small differences in size among most chromosomes during mitotic metaphase, SC karyotypes helped to increase the resolution in the total length and position of centromeres. In this way, a comparison was made between mitotic and meiotic karyotypes in *O. niloticus* and *O. karongae*.

O. karongae has a karyotype composed of 2n=38 chromosomes, while the other species studied were 2n=44. This difference in chromosome number is reflected in the increase in size of three chromosome pairs in *O. karongae* (the kind of fusions observed by SATA probes is discussed in 3.5.7), demonstrated to involve LG1 in one case. This LG appeared on a small chromosome in *O. niloticus* FISH mapping, whilst on *O. karongae* it appeared in chromosome 2. It is evident that a major arrangement has happened very recently in evolutionary terms when comparing *O. karongae* with *O. mossambicus* and *O. mortimeri*, due to the phylogenetic proximity between these three species (Sodsuk *et al.,* 1995). Pressuming that LG1 and LG3 are the main players in sex determination as observed in *O. niloticus* (XY) and *O. aureus* (WZ) respectively (Lee *et al.,* 2003; Lee *et al.,* 2004), a chromosomal rearrangement in *O. karongae* (WZ) including one of the putative linkage group involved in sex determination, could suggest that the sex-determination system is also evolving in tilapia species very rapidly and with potential change in the

master sex-determination locus in use. This sex chromosomes rearrangement has been observed in close related species of stickleback (Peichel *et al.,* 2004). Moreover, in tilapia hybrids has been proposed a transition between sex-determining systems XY/ZW in *O. aureus* X *O. niloticus* hybrids (Ezaz *et al.,* 2006), and no evidences of unpaired bivalents in *O. niloticus* X *O. karongae* hybrid siblings despite the chromosome rearrangement in *O. karongae* (Harvey *et al.,* 2002a).

There is a general pattern of a tendency in a decrease of chromosome number in tilapia species with the well documented speciation (Kocher, 2004) as observed in the lower chordates (Thiriot-Quievreux, 1994), although there are some exceptions within the ray-finned fishes showing a phylogenetic conservation of chromosome numbers (Mank *et al.,* 2006). In the present research, *O. karongae* represents an example of evolutionary divergence of species among the genera *Oreochromis,* leading into a reduction of chromosome number even in close related species.

Going back to the mitotic physical map for *O. niloticus* (Figure 3.35; Martins *et al.,* 2004; Oliveira and Wright, 1998), there are some differences in the order/total length of the submetacentric chromosomes (Chromosome 6, 11 and 15) compared to their position on the SC karyotype (Figure 3.33) (chromosome 5, 7 and 11). This could perhaps be evidence of recombination influences affecting the size of chromosome arms and supporting the idea that a more accurate representation of cM distances is achieved by analysis of SCs during pachytene stages, as suggested for zebrafish (Wallace and Wallace, 2003), humans (Collin *et al.,* 1996) and rice (Cheng *et al.,* 2001). Another

reason for these differences could be just the result of having difficulties sorting the small chromosomes from mitotic stages.

In *O. karongae* was also demonstrated to have some differences in the distribution of some of the small chromosomes when comparing SC karyotype arrangement stained by DAPI against mitotic karyotype arrangement stained by PI and hybridised with SATA sequences. It was clearly identified on SC spreads, three submetacentric chromosomes (chromosome 4, 6 and 11) whilst on mitotic spreads, there were three clear submetacentric chromosomes (4, 6 and 11) and other three in the borderline between subtelocentric/submetacentric chromosomes (8, 14 and 19). When comparing the SC spreads against mitotic spreads both stained by DAPI, three submetacentric chromosomes were recognised all the time, being also easier to identify chromosomes by DAPI than PI stain. It is true that SATA signals helped to localise with more accuracy the centromeres than just DAPI or PI stain during mitotic stages. However, the resolution of chromosome tips (sharpness) under PI stain (red fluorescence) against the black background was not optimal for FIpter measurements. This problem is easier to overcome under DAPI stain (blue fluorescence) or by changing the picture to black/white. The lack of accuracy is compounded by the very small size of the chromosomes. This could be improved by analysing SC bivalents.

In the end, these differences could be not really significant and could be only explained again for the lack of accuracy on the small mitotic chromosomes, improving results when comparing with SC bivalents.

Another important observation was a constant area of weaker DAPI staining appearing near to the middle of chromosome 2 in meiotic spreads of *O. niloticus* and on the equivalent chromosome 5 in meiotic spreads of *O. karongae*. In both cases this was followed by a stronger bright dot like the one observed from centromeres. This characteristic was quite clear and consistent among different fish. At the same time, using PI staining, a single submetacentric, chromosome 6, was detected on mitotic spreads from *O. karongae* with brighter p arms and from *O. niloticus* at least one pair of submetacentric small chromosomes was also seen with brighter p arms. Then the presence of heterochromatin accumulation on these specific sites was suggested, as is often observed on p arms of subtelocentric or acrocentric chromosomes (Oliveira and Wright, 1998). The same authors also mention that SATB repeats strongly hybridised onto a single chromosome pair that could lead to the association of this with chromosome 4 on Martins' physical map.

3.5.6 Differences observed between the two homologues of chromosome 1 in *O. niloticus*

All the chromosome spreads analysed from *O. niloticus* were from phenotypic and genotypic XY males. Because of the relatively small and equal sizes of most of the chromosomes, only chromosomes 1, 2 and 6 from mitotic spreads could be recognised (Oliveira and Wright, 1998). From these three recognisable chromosomes, only chromosome 1 (one of the putative sex chromosomes) is large enough to possibly detect any constant pattern from DAPI or PI stain.

As observed in Figure 3.36, in almost all chromosome preparations analysed (at least 5 XY males Nile tilapia and one individual from each of the other seven species) it was possible to detect different patterns between the two copies of chromosome 1. Probes hybridised along chromosome 1 also served as references for characterising the constricted and brighter areas observed.

The identified chromosome 1 type A presented two constrictions (and/or negative DAPI band), one clearly localised slightly before the physical localisation of marker *OniY*227 at Flpter= 0.56 and the other close to the area where the insulin gene appears at Flpter= 0.70. This second constriction also showed a brighter zone towards the telomeres of the long arm. Chromosome 1 type B presented also two constrictions but in different locations, one constantly observed close at Flpter= 0.24 where the physical localisation of DMO (dmrt4) gene is found, and the second one at Flpter= 0.81 close to the location of marker GM354 where the brighter zone begins towards the telomeres of the long arm.

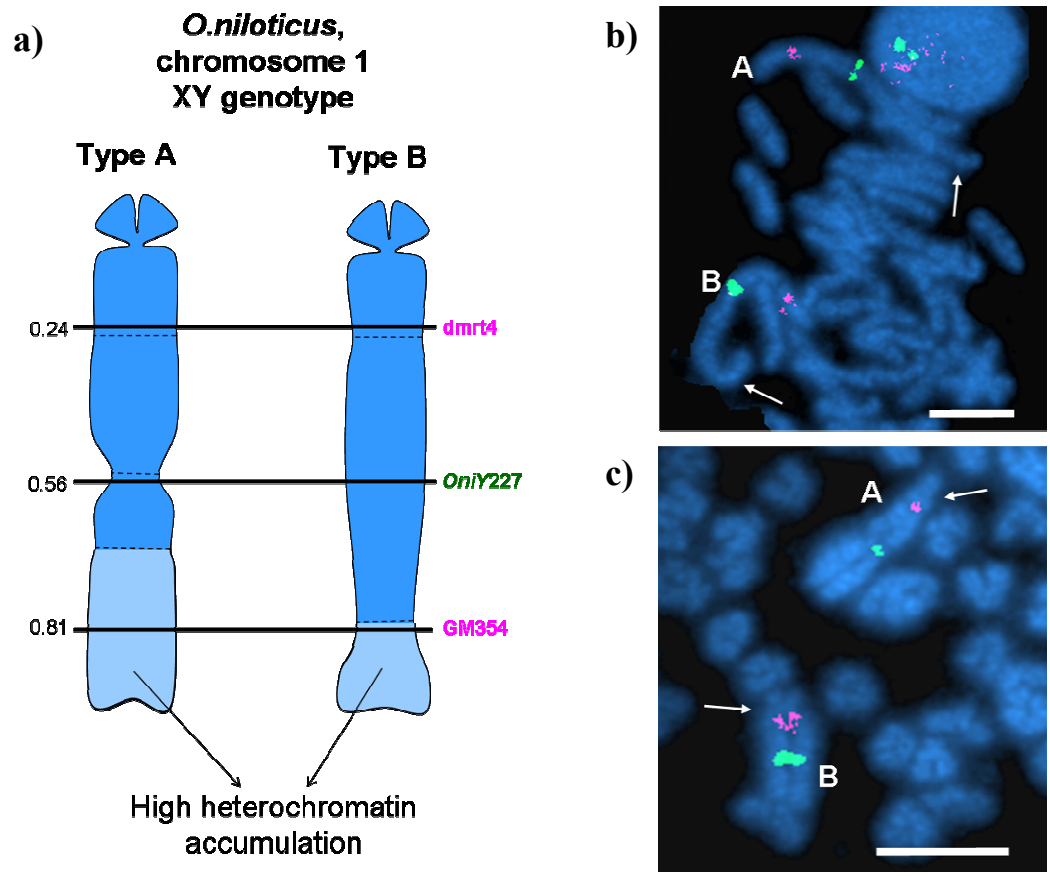

Figure 3.36 Homologous mitotic chromosome 1 from male XY *O.niloticus* visualised by DAPI staining. Markers GM354 (red colour, close to telomere on q arm), *OniY*227 (green colour) and dmrt4 (red colour, close to centromere on q arm) are presented as references. a) Graphic representation of homologous chromosomes, type A and B, illustrating the different morphologies and areas of high heterochromatin accumulation; b) prometaphase stage identifying type A and B chromosomes, also showing hybridisation from GM354 and *OniY*227 markers; c) metaphase stage identifying type A and B chromosome, also showing hybridisation from *OniY*227 and dmrt4 markers. Arrows on (b) and (c) indicate centromere position. Notice the differences in thickness and brightness along the largest chromosomes in picture (b). Scale bar = 5µm.

Several authors (Majumdar, 1986; Martins *et al.,* 2004; Phillips, 2001) failed to identify significant banding patterns in tilapia chromosomes, using traditional staining techniques (such as Giemsa, C-banding and DAPI). Nevertheless, Oliveira and Wright (1998) observed some regular banding following restriction endonuclease treatment and C-banding. Chromosome bands were attributed to heterogeneous composition of heterochromatin in tilapia, differentiated into regions along the chromosomes with distinctive structural properties. It was hypothesised that chromosome replication in fish is

temporally clustered into early and late replicant units, showing a distinct replication band pattern on each chromosome (Gold and Li, 1991). Not only were some differences in the heterochromatin (by DAPI brightness) observed on the largest chromosome here, but also in its general morphology.

There are some pieces of evidence supporting these findings described by Chen (1971), who observed that two subtelocentric chromosomes (presumed X chromosomes in an XY system) in the killifish species *Fundulus parvipinnus* and *F. diaphanus* were marked by a constriction close to the centromere. Unfortunately this was not followed up by directly comparing males and females.

In the other seven species studied, it was not possible to properly compare phenotypic males and females from all the species due to the lack of sharpness on chromosome spreads, but at least the males of different species showed the differences in morphology on chromosome 1 as described before (independent of the sex-determining system described for each species, XY or WZ), including the brighter DAPI staining at the end of the q arms.

De Almeida-Toledo *et al.* (2001b), identified the X chromosome due to a heterochromatinisation process in the electric eel *Eigenmannia virescens* when comparing several closely related species; but much of the evidence in WZ and even XY systems in fish in early stage of sex-chromosome differentiation has involved heterochromatinisation of the Y or W chromosomes (de Almeida-Toledo and Foresti, 2001a), at least in neotropical freshwater fish.

Here it is proposed that the polymorphism observed under DAPI staining between the pair of chromosomes 1 in XY chromosome plates could be related

to sex-linked inheritance. Nevertheless and more important, these observations have to be consolidated with further comparisons among XX and YY karyotypes after proper progeny testing. By this means, it would be possible to determine whether type A or B carries one of the putative sex-determining loci and how it relates with the unpairing region observed in pachytene spreads and the 13cM sex-expected range described in LG3. Such a procedure would also allow testing as to whether the observations were just artefacts or rare features.

3.5.7 Cytogenetic analysis of repetitive DNA (SATA) and telomeric sequences $(TTAGGG)_n$ in tilapia species

It is important to mention that FIpter comparisons from total size and centromere position in *O. niloticus* and *O. karongae* became puzzling between DAPI stained spreads (A/T staining; Invitrogen, product information) and PI stained spreads (DNA and RNA staining with little sequence preference; Invitrogen, product information) as discussed in previous sections. It is possible that the different treatments of the chromosomes (during FISH procedures) and the staining itself resulted in slight differences reflected in the classification. Nevertheless, the comparison with meiotic spreads helped to correct the chromosome classification from both species.

On chromosome 1 in Nile tilapia, previous studies (Chew *et al.*, 2002) reported telomeric sequences $(TTAGGG)_n$ found interstitially at two loci, supporting the hypothesis of fusion of three acrocentric chromosomes. Such an observation could account for the reduction in chromosome number in Nile tilapia (2n=44) compared to ancestral African cichlids (2n= 48), e.g. *O. alcalicus*

(Park, 1974). See section 3.5.5 for a previous discussion on proposed chromosome reduction.

It is presumed that 100kb BAI31-insensitive EcoRI (telomeric sequences) fragments are present interstitially on chromosome 1 in Nile tilapia as observed by Chew *et al.* (2002). Although relative distances were not reported for these two interstitial telomeric signals, Flpter measures taken from the images presented in Chew *et al.* (2002) revealed positions for the first and second interstitial signals of Flpter=0.45 and 0.62 respectively. Compared with the cytogenetic map from this project, these two signals would be located between Clcn5 (Flpter=0.34) and UNH115 (Flpter=0.47) for the first signal and for the second one, between *OniY*227 (Flpter=0.56) and *OniY*382 (Flpter=0.69).

It is also known the telomeric-associated sequences (TAS) are middle repetitive DNA sequences that are adjacent to the telomeric repeat tract (Chew *et al.,* 2002) and is suggested that they are involved in the chromosomal fusion of chromosome 1 during karyotype evolution, as could have happened in *O. karongae* on the middle sized chromosomes 2, 3 and 4.

Nevertheless, interstitial signals from telomeric sequences were not clearly seen in the present hybridisations from either species, *O. niloticus* or *O. karongae,* despite the use of low stringency conditions (35% formamide), and using oligomers of telomeric repeats as Chew *et al.* (2002) previously reported. These results raise the question as to whether these are real interstitial telomeric signals or are hybridisations caused by the high heterochromatin accumulation, the non specific low stringency required to visualise the signals and the nature of the probes (PCR amplifications with several base errors that

lead to a non-specific hybridisation). In *Salmo salar*, Abuin *et al.* (1996) obtained interstitial signals from telomere sequences by FISH using oligomer probes while Perez *et al.* (1998) did not get any interstitial signal using cloned salmon telomeres. Another reason could be polymorphism within large blocks of interstitial telomeric DNA sequences. This may lead to intraspecific differences in interstitial hybridisation sites in Nile tilapia. (Wiley *et al.,* 1992) gave evidence of this kind of polymorphism in hylid frogs, attributing this phenomenon to unequal crossing over, sub-microscopic deletion or differential amplification.

In the other species, identification of SATA sequences by FISH were only observed in the centromeric region of each chromosome in the species *O. aureus, O. mossambicus, O. mortimeri, O. niloticus, S. galilaeus, T. rendalli* and *T. zillii*, with no other interstitial signals.

In *O. karongae*, interstitial SATA signals were detected on chromosomes 2, 3 and 4 at different relative positions each. Because of the reduction in number of chromosomes from 2n= 44 to 2n=38 when comparing other close related *Oreochromis* species, it is evident that fusions have happened very recently in evolutionary terms for this species as mentioned in the previous section.

These interstitial signals demonstrated a telomere-telomere fusion in chromosome 2 (subtelocentric), including LG1 (see Figure 3.19); a centromere-telomere fusion in chromosome 3 (Subtelocentric); and a centromere-telomere fusion in chromosome 4 (submetacentric).

The recurrence of Robertsonian translocations (centromere-centromere fusion) as suggested previously in this species for the identified submetacentric chromosome 4 (chromosome 3 in Harvey *et al.,* 2002a) is debatable due to a clear short gap observed between the two SATA signals on this chromosome. This suggests that a better model may be centromere-telomere fusion in chromosome 4. However the data presented in this thesis agrees with the analysis of Harvey *et al.* (2002a) that chromosome 2 and 3 are Robertsonian translocations.

Earlier, it was proposed that dual FISH could be employed to refine the assignment of LG1 to one of the small chromosomes. A similar approach could be used to verify mapping of LG1 to chromosome 2 in *O. karongae*. Although the evidence presented here seems solid, chromosome 3 has also great morphological similarity to chromosome 2 but they can be differentiated by the location of the signals from the SATA probe on the q arms.

3.5.8 Conclusion

The *Oreochromis niloticus* and *O. karongae* physical maps presented in this chapter represent the most precise and complete physical maps constructed until now. Moreover, detailed karyotypes from both species based on SC spreads have been developed.

The advantage of using nick translation for labelling of BAC probes in tilapia was demonstrated. Increased resolution of FISH on SC spreads (compared to mitotic spreads) and the potential utility these may have during the assignment of linkage groups, had been also shown. The combination of

different molecular and cytogenetic techniques was confirmed to have great utility in the process of physical mapping.

The search for the sex determination locus on LG3 in *O. niloticus* was narrowed down to 13 cM, located at the first 13 cM of the linkage group and at the edge of the unpaired region according to the physical map.

Linkage group 1 in *O. niloticus*, localised on a small submetacentric chromosome, is suggested to refer to chromosome 4, 5 or 7 represented in Martins' ideogram (2004).

The other six species studied with 2n= 44, demonstrated a conserved karyotype distribution for two of the main linkage groups involved in sex determination in tilapia. Meanwhile, in *O. karongae* (2n=38) it was shown, by SATA and UNH995/UNH104 hybridisation, that LG1 has been re-arranged into the subtelocentric chromosome 2 as a result of a telomere-telomere fusion.

All the tilapia species analysed showed no obvious morphology differences between the two putative sex chromosome pairs (chromosome 1 and a small submetacentric chromosome in *O. niloticus* or chromosome 2 in *O. karongae*) suggesting a nascent Y chromosome in an early stage of differentiation despite chromosome fusions and/or different sex determination systems previously described.

Six LGs were assigned to chromosomes in Nile tilapia, and it is suggested to continue further linkage assignments with the rest of the linkage groups to facilitate the identification of all the small chromosomes.

Considering that sex-determination systems can evolve very quickly even in close related species and that the master sex-determination genes are not conserved between divergent taxa, it would be quite acceptable to consider two linkage groups playing the main role in sex determination (as demonstrated with LG 1 and 3). So more attention on detailed molecular and cytogenetic studies are required on LG1 as has been done for LG3. *O. karongae* (WZ) offers now the possibility to extend the studies on LG1-chromosome 2 and to find out whether this LG involves the main sex determination gene or if an epistatic interaction is also involved as suggested in *O. aureus* (Lee *et al.*, 2004) with the presence of a master gene on LG3, chromosome 1 (Cnaani *et al.,* 2007).

Finally, it is also proposed to extend further studies, comparing males and females FISH-SCs on chromosome 1 and the small chromosome (LG1) in *O. niloticus*, and chromosome 1 and 2 in *O. karongae*, to localise the specific physical area of suppression in recombination as linkage groups 1 and 3 predicted. As a consequence, it will narrow the putative areas containing the sex-determination loci.

Chapter 4 MICROSATELLITE ISOLATION FROM CHROMOSOME 1

4.1 Summary

In order to find polymorphic markers to aid the comparison of physical and linkage mapping of chromosome 1, fifteen chromosome 1 tilapia BAC clones (with an average insert of 105 kb each) previously identified, were screened for microsatellites. Some of these BAC clones contained sequences of interest already identified as sex-linked AFLPs, dmrt 1 and dmrt 4 genes and several SINEs.

DNA fragments from digestion with *Sau*3AI were selected in the range of 200-600bp and cloned into a suitable vector/host system. Four tandem repeat probes were designed and labelled with ^{32}P for radioisotopic-based screening. Positive clones were sequenced and primers were then designed and tested for amplification and polymorphism.

After the screening of 2,000 subcloned colonies, five microsatellite sequences were demonstrated to amplify properly from positive controls and genomic DNA but only one (associated with dmrt 1 gene containing BAC) was confirmed to be polymorphic, co-dominant and informative in specific fish families used in this study. Additionally, twelve sub-cloned sequences showed homology with several tilapia genes and DNA sequences published in the NCBI Genbank, opening the possibility for further research to confirm the presence of these homologous sequences and the anchoring of physical and linkage map on chromosome 1.

4.2 Introduction

4.2.1 Development of new microsatellites

Microsatellite loci consist of multiple copies of tandemly arranged simple sequence repeats (SSRs) that range from 1 to 6 base pairs. They have already been identified in many fish species, including salmonids such as Atlantic salmon, rainbow trout, brown trout for application in population genetics (O'Connell and Wright, 1997; Slettan A *et al.,* 1997) and have been estimated to occur as often as once every 10 Kb in fishes (O'Connell and Wright, 1997; Wright and Bentzen, 1995). This makes them very useful for genome mapping studies. These markers are present in both coding and noncoding regions but show greater probability to be type II markers (e.g. associated with anonymous genomic segments) (Zane *et al.,* 2002). Despite their wide and relatively homogeneous distribution, it is not expected that genome size and the number of microsatellite loci should be related, because the amount of coding and non-coding DNA is generally independent of the genome size (Neff and Gross, 2001).

Another important characteristic is that this kind of marker generally has to be isolated *de novo* for new species (Zane *et al.,* 2002) but once they have been identified, they can often be amplified in related species (Varshney *et al.,* 2005). Nevertheless, the efforts put into the development of microsatellites are generally worthwhile because of their high degree of length polymorphism, generating multiple alleles across different species or even within a fish population, and their co-dominance, segregating in most instances, among offspring (Liu and Cordes, 2004a).

Discovery of microsatellites markers is slow relative to techniques such as Amplified Fragment Length Polymorphism (AFLP). For the rapid construction of a genetic map suitable for QTL analysis in any particular cross, AFLP may be the faster route but is balanced by the potential risk of more scoring errors (Kocher *et al.,* 1998) and reduced informativeness. AFLPs are predominantly dominant markers and exhibit some reproducibility problems during amplification. Single Nucleotide Polymorphism (SNP) markers, the most abundant polymorphic marker in organism, have the potential to identify type I markers, in contrast to microsatellites, but SNPs have current disadvantages of limited number of alleles (usually two) (Liu, 2004) and requirement for more specialised equipment for their identification and scoring (Vignal *et al.,* 2002).

Microsatellites provide then, a stable framework for population studies, paternity analysis and the construction of genetic maps to enable subsequent positional cloning of several genes (Agresti *et al.,* 2000; Lee *et al.,* 2005; Liu and Cordes, 2004a; Schlotterer, 2000). The use of enrichment protocols during the isolation process is often applied mainly when a low probability of finding specific microsatellite exist (Neff and Gross, 2001; Zane *et al.,* 2002).

$(CA)_n$ is one of the most common microsatellite repeat motif encountered in vertebrates, been observed in 98 species of fish, reptiles, amphibians, birds and mammals (Neff and Gross, 2001). In tilapia, $(CA)_n$ microsatellites are also common (Lee and Kocher, 1996). Many were found during the screening of three tilapia BAC libraries (96% CA repeats from the total clones isolated) (Carleton *et al.,* 2002). A clear example of their abundance is that during the construction of a second generation genetic linkage map, 62 new

microsatellites were isolated after the screening of a tilapia genomic BAC library with the $(CA)_{10}$ probe (Lee *et al.,* 2005).

An important efficiency aspect to consider during the microsatellite isolation process against the total number of useful markers expected is the finding of false "positive" clones which have no repeat, insufficient flanking sequence, too short repeats, duplicate clones and clones whose repeats are too complex, for mentioning some of the most common observed (Carleton *et al.,* 2002).

4.2.2 Basic comparative genomic tools

To date, the full understanding and speed of development of aquaculture genomics, including tilapia species, depends on information from well-studied species such as human, mouse and zebrafish (Liu and Cordes, 2004a). One of the most commonly used tools for DNA and protein comparisons is found at the National Centre for Biotechnology information (NCBI) website (http://www.ncbi.nlm.nih.gov/BLAST/) with the application of basic local alignment search tools (BLAST) and Position-Specific Iterated-Blast (PSI-BLAST) tools developed by Altschul *et al.* (1997).

Supported by genetic sequence databases (such as GenBank, collection of all publicly available sequences), these bioinformatic tools provide the basis for comparative genomics for finding orthologous and homologous genes, highly conserved repetitive and non-repetitive regions, vector contaminations, restriction maps, find sequences with gene expression and search for SNPs within other applications.

4.2.3 BAC clones selected from chromosome 1

Considering the utility and the power of polymorphic microsatellite markers, a shortcut strategy was applied in this research for the identification of genetic markers that could be tightly linked to the sex determination locus on the putative sex chromosomes (chromosome 1), as proposed by several authors (Campos-Ramos *et al.,* 2001; Carrasco *et al.,* 1999; Harvey *et al.,* 2002b). This strategy consisted of pooling and screening tilapia BAC clones previously identified by southern blot and FISH techniques that contain sequences from chromosome 1.

Similar strategies have been applied in the greater white-toothed shrew for the isolation of Y chromosome microsatellites from BAC clones, overcoming the difficulties of marker isolation and low levels of nucleotide diversity compared to the human genome (Handley and Perrin, 2006).

It has been suggested that the sequence differences between X and Y chromosomes in Nile tilapia are largely confined to non-coding regions (Mair *et al.,* 1997). By analysis of repetitive DNA sequences, these differences were visualised by fluorescent *in situ* hybridisation (FISH), roughly identifying an area of differentiation on the second half of the long arm towards the telomere on chromosome 1 (Harvey *et al.,* 2003b). This region is also characterised by a unique accumulation of short and long interspersed repetitive elements (Oliveira *et al.,* 1999, 2003) and the presence of sex linked markers (Ezaz *et al.,* 2004b).

The present strategy aimed to identify a series of markers distributed along the chromosome 1 for further comparison of physical and linkage

distances. It was recognised that this was a challenging approach given the extensive repetitive DNA sequences expected to be present in the region of interest; nevertheless, a microsatellite approach was the only feasible option available due to the time and cost constrains. BAC clones from the tilapia library (Harvey S.C. and Boonphakdee C., unpublished observations) held at the University of Stirling (USTI-02TI with 105kb inserts), which were localised on a preliminary physical map by *in situ* hybridisation on chromosome 1 in tilapia were used. Most of them were localised on the second half of the large arm on this chromosome. The 15 BAC clones contain Sex Linked AFLP Markers (SLAMS) (Ezaz *et al.,* 2004b), SINE repeat sequences (Harvey *et al.,* 2003b), and Dmrt and Sox genes (Boonphakdee, 2005) and some others not characterised but identified by probes containing microdissected subtelomeric regions from chromosome 1 (Harvey *et al.,* 2002b).

4.2.4 Objectives

The research developed in this chapter was focused on four main points:

- To find new microsatellite markers along the putative sex chromosome 1.
- To identify sex linked markers by comparison of physical and linkage distances.
- To anchor the existing LG3 (assigned to chromosome 1) with the resultant markers.

- To identify homologous sequences from other organisms by BLAST for the potential assignment of synteny genes to chromosome 1 (from type I markers).

4.3 Materials and Methods

As an overview, fifteen BAC clones previously hybridised to Chromosome 1 were digested with *Sau*3AI restriction enzyme to produce fragments with cohesive (sticky) ends. The resultant fragments were size selected (200 to 600bp) and ligated into pBluescript vector for transformation into supercompetent cells (sub-cloning). The resultant cells were screened with radiolabelled probes (containing typical repetitive motifs, see Figure 4.1) for detection of positive clones by colony screening techniques, and positive clones sequenced for characterisation of repetitive motifs and design of flanking primers. Once the set of primers were confirmed to amplify the expected fragment from the sub-cloned construct, they were tested on XX and XY genomic DNA, and subsequently on the fifteen selected tilapia BAC clones in order to identify the original BAC containing the putative microsatellite marker. Finally sequences were compared (by BLAST) against databases on the public domain to find any relevant similarities, and polymorphism checked with the use of different Nile tilapia families. The resulting polymorphic sequences were then used for further linkage analysis.

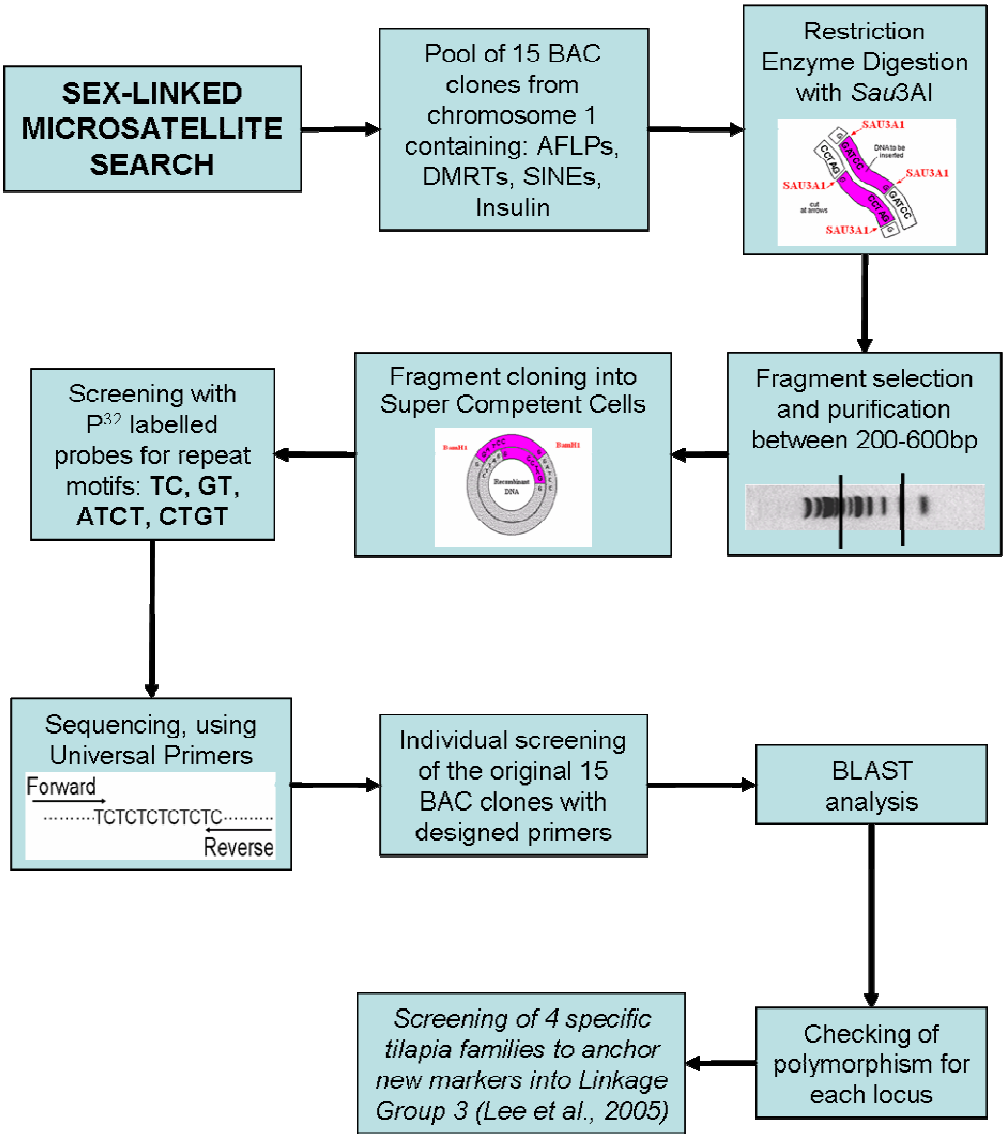

Figure 4.1 Flow diagram representing the steps covered during the development of sex-linked microsatellites. The selected BAC clones previously detected on chromosome 1 by FISH are the starting point; the new marker(s) in the end were screened together with markers from LG3 for any possible new anchoring.

4.3.1 Pool of selected clones

BAC clones already identified as hybridising to chromosome 1(Table 4.1) from the Stirling BAC library (USTI-02TI) were grown individually. BAC DNA was extracted from each of the cultures (see section 2.4.1) and resuspended in TE buffer to a standardised concentration of 100 ng/µl. Equal amounts (2

µl/each) of the fifteen extracted plasmids were pooled into a single tube and gently mixed.

Table 4.1 BAC clones hybridised on chromosome 1 and pooled for the screening of microsatellites. Map location of markers refers to previous observations from Chapter 3, in FIpter.

BAC CLONE	WELL ID	CLONE IDENTITY	MAP LOCATION	AUTHOR(S)
1	28N20	Dmrt 1 gene	Chrom. 1, FIpter 0.85 (see Figure 3.9)	Boonphakdee, 2005
2	139F12	?	Chrom. 1	Harvey *et al.*, 2003b
3	102M10	Dmrt 4 (DMO) gene	Chrom. 1, FIpter 0.24 (see Figure 3.11)	Boonphakdee, 2005
4	130I9	?	Chrom. 1	Harvey *et al.*, 2003b
5	75I10	Insulin and *OniY*382	"Chrom. 1", FIpter 0.69 (see Figure 3.11)	Boonphakdee, 2005; Ezaz *et al.*, 2004b
6	39G12	Dmrt4 (DMO) gene	Chrom. 1, FIpter 0.24 (see Figure 3.11)	Boonphakdee, 2005
7	72B20	*OniY*227	Chrom. 1, FIpter 0.56 (see Figure 3.11)	Ezaz *et al.*, 2004b
8	137C10	?	Chrom. 1	Harvey *et al.*, 2003b
9	115C16	Sine 4	Chrom. 1, telomere of long arm	Harvey *et al.*, 2003b
10	137D12	?	Chrom. 1	Harvey *et al.*, 2003b
11	117N18	Sine 2	Chrom. 1, FIpter 0.69 – 0.81 (see Figure 3.9)	Harvey *et al.*, 2003b
12	17L4	Sine 8	Chrom. 1, FIpter 0.69 – 0.81 (see Figure 3.9)	Harvey *et al.*, 2003b
13	119C17	Possibly Sine 1 or 7	Chrom. 1, FIpter 0.69 – 0.81 (see Figure 3.9)	Harvey *et al.*, 2003b
14	126N3	Sine 3	Chrom. 1, middle point of long arm	Harvey *et al.*, 2003b
15	124F18	Sine 6	Chrom. 1, telomere of long arm	Harvey *et al.*, 2003b

? = BAC clone not characterised but hybridised on chromosome 1.

4.3.2 Sub-cloning of selected fragments

Before re-digestion, several tests using genomic DNA from an XY male tilapia were digested using six enzymes to see which one led to the best homogeneous smear of fragments size between 200 to 600bp. The enzymes tried, with their respective cleavage sites were *Nde*II ('GATC), *Sau*3AI ('GATC), *Mse*I (T'TAA), *Cla*I ('CGAT), *Hae*III (GG'CC) and *Alu*I (AG'CT). They generated

cohesive or blunt ends and each of them were applied according to the manufacturer recommendations (New England BioLabs, UK).

Digestions were checked by electrophoresis on a 1.5% agarose gel. The enzyme generating the best smear (*Sau*3AI) was chosen then to digest the pool of plasmids. For the preparation of the subcloning vector pBluescript II KS-, an enzyme that generates compatible ends with the target fragments was chosen (*Bam*HI). Once digested, dephosphorylation was carried out with Shrimp Alkaline Phosphatase (SAP) to decrease the possibility of self ligation (see section 2.4.2).

The pool of BAC constructs was digested with the selected enzyme (*Sau*3AI). To isolate the fragment sizes desired from the digested smear, 13 µl of the sample (20 ng/µl) was run on a 0.8% low melting agarose gel and the specific fragment size range was extracted and purified from the gel using a concert rapid gel extraction system kit following manufacturer recommendations (Life Technologies, UK). This time, two µl of the resultant product was run on 1.5% agarose gel to confirm the success of the fragment selection.

In order to optimise the ligation technique and increase the chances of getting a wide screening of the fragments generated, three different vector:ligation ratios (3:1, 1:1 and 1:3) were tried along with positive and negative controls (Table 4.2) before running the transformation on a larger scale.

As described in section 2.3.2, quantification of selected DNA fragments and vector was done with the use of 6X loading buffer with reduced dye (1 part

6X load dye + 19 parts of 15% of Ficoll diluted in water). A 1 Kb ladder with the special loading buffer was loaded at four different concentrations on a 1.5% agarose gel (1.0 µg, 0.5 µg, 0.25 µg and 0.125 µg). Three different amounts of sample from selected DNA fragments and cut vector were loaded using the same loading buffer. Concentrations were then estimated by comparing band intensity from DNA fragments and vector with the known 1 Kb ladder bands.

The transformation procedure was done by using the supercompetent cells kit (SCS1; Promega, UK) which are optimised to ensure a high transformation rate. One µl (10 ng/µl) of the newly created construct solution was added into a 100 µl of supercompetent cells and 1.7 µl (25 mM) β-mercaptoethanol. The resultant suspension with transformed cells was spread on 27 cm^2 LB-agar plates prepared with 80 µg/ml methicillin and 20 µg/ml ampicillin for selection of cell resistance, and 80 µl of X-gal (20 µg/ml) and 40 µl IPTG (100 mM) for white/blue (vector with insert or without insert respectively) colony colour differentiation. An individual LB-agar plate was used for each of the three vector:ligation ratios tested (see details in section 2.4.3 and 2.4.4).

The vector:ligation ratio presenting the highest number of total colonies (counting white and blue colonies) was chosen to be practiced in a larger scale. Figure 4.2 represents the observations from the subcloning optimisation process. For this purpose, four different scenarios were obtained from the seven master mixes prepared, leading to the estimation of transformation efficiencies (% of white colonies).

Table 4.2 Optimisation of master mix for ligation purposes using different vector : insert ratios.

Components	Negative control 1	Negative control 2	Positive control 1	Positive control 2	Vector/ Insert 3:1	Vector/ Insert 1:1	Vector/ Insert 1:3
Ligation Buffer (10X)	1µl	1µl	1µl	1µl	1µl	1µl	1µl
T4 DNA Ligase (100u/µl)	0.5µl	----	0.5µl	0.5µl	0.5µl	0.5µl	0.5µl
Vector (50ng/µl; cut & dephosphorilated)	0.5µl	0.5µl	----	----	1µl	1µl	0.5µl
Insert DNA (10ng/µl)	----	----	----	----	1.6µl	5.0µl	8.0µl
Uncut Vector (50ng/µl)	----	----	0.5µl	----	----	----	----
Cut Vector (50ng/µl)	----	----	----	0.5µl	----	----	----
Molecular Grade Water	8µl	8.5µl	8µl	8µl	5.9µl	2.5µl	----
Total Reaction Volume	10µl	10µl	10µl	10µl	10µl	10µl	10µl

4.3.3 Detection and design of microsatellite primers

Due to the higher sensitivity of radiolabelling in comparison with chemifluorescence detection, the screening of positive clones onto the membrane was done using probes radio-labelled with γ ^{32}P-ATP (ICN 70072; 4,500 Ci/mmol; 10 µCi/µl) at the 5' end. During the 5'end-labelling procedure, T4 polynucleotide kinase (10 u/µl) was used according to manufactures conditions. The four specific microsatellite oligo probes are detailed in Table 2.6. Equal molar ratio of each probe were combined and labelled together as described in Table 2.7, incubated for 30 min at 37°C and subsequent enzyme inactivation for 20 min at 95°C.

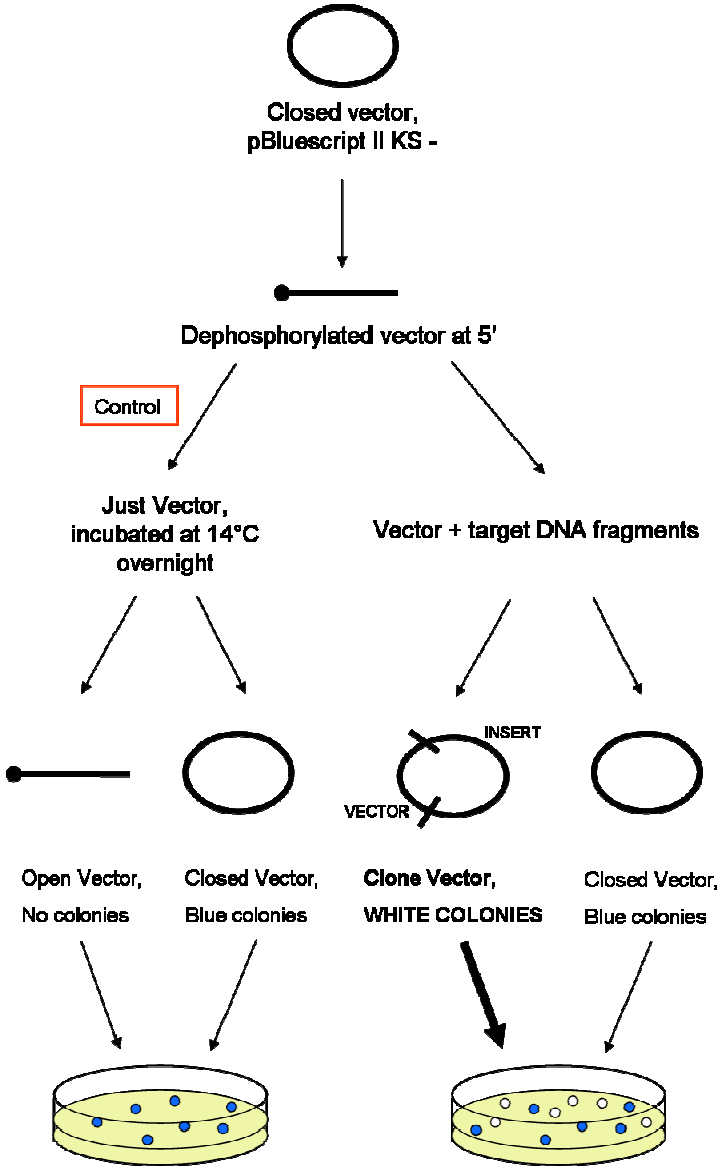

Figure 4.2 Graphical procedure for the subcloning of selected fragments. Target DNA fragments came from 15 selected BAC clones containing sex-related sequences hybridised on chromosome 1 in Nile tilapia.

The identification and isolation of positive clones was completed within three days of growing of recombinant bacteria on a large petri dish (500 m^2) to reduce contaminant/satellite colony development. Hybond-N+ membranes were used to lift and fix the colonies from the plate. It is important to highlight that the large petri dishes with LB-agar were complemented only with antibiotics (80%

methicillin / 20% ampicillin), but not with X-gal or IPTG as it was not required for colony screening with probes. A temperature of 60°C for 3.3 h pre-hybridisation and 53°C for overnight hybridisation was used. Short sequences were stripped off by applying a series of post-washings as described in section 2.4.6. Membranes were exposed overnight at -70°C to an autoradiograph film with intensifying screen for the detection of positive colonies.

Positive dark spots were matched with the original colony petri dish and selected colonies picked up with toothpicks to be transferred onto new small LB-agar petri dishes for blue / white differentiation checks. Confirmed white colonies were finally arrayed onto 96 well plates containing LB-broth with antibiotics 15% glycerol for an overnight incubation at room temperature.

These selected colonies were arrayed into 96 well plates according to the hybridisation strength (strong, medium and weak signal). A second round of hybridisation/isotopic screening was employed to confirm positive signals before sequencing. This reconfirmation was carried with the lifting and fixing of arrayed colonies onto new Hybond-N+ membranes as described above, with the difference that colonies were lifted directly from the LB-broth onto the membrane with no previous growth on LB-agar using a 96 pin replication tool.

After standard plasmid extraction, the confirmed positive clones containing sub-cloned fragments were sequenced in both directions on an ABI Prism 377 system.

Using a combination of bioinformatics software -Chromas v. 1.15 (http://www.technelysium.com.au/chromas.html), BioEdit v. 7.0 and DnaStar software v. 7.0- forward and reverse sequences were aligned to create a

consensus sequence. Once the consensus was determined, vector fragments (pBeloBAC11 and pBluescript II KS-) and gDNA from *Escherichia coli* were trimmed and the resulting sequences screened for potential repeats with Tandem Repeat Finder (TRF) v. 3.0 software.

Identified microsatellites were located along each sequence and appropriate primers designed using the on-line Primer3 v. 0.4 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) applying default parameters (except that a product size of 200bp was selected). Tandem repeats close to the end or missing a flanking sequence were not suitable for primer design.

4.3.4 Sequence BLAST in GenBank

All sequences were subjected to BLASTn analysis. The resulting Blast helped to identify sequence homologies with other species and possibly associate the developed microsatellite sequences in this research with other genes, markers or sequence clusters (such as dmrt 1,2,3; Boonphakdee, 2005) that may be present on chromosome 1 and more specifically, linked to the sex determination locus. Beside this, from the sequences with no suitable primers to design, BLASTn results could lead to the finding of missed flanking regions by walking either upstream or downstream of the homology sequence.

4.3.5 Validation of new microsatellites

New primers designed from the sub-cloned fragments were validated at several levels. They were tested first by PCR on the sub-cloned constructs, second on genomic DNA from an ordinary male and female tilapia (XY and XX), third on the fifteen BAC clones from which the fragments were selected originally, and fourth on one non-related BAC clone as a negative control (containing brain aromatase gene). Correct amplification under optimised standard-based conditions and the size expected were taken in consideration to take every set of primers forward. Primers tested with the original fifteen BAC clones were expected to have a unique amplification from at least one of these BAC clones. Successful primers were then designed for tail labelling, using M13 universal primer 5' end labelled with one of three optional fluorescent dyes in order to read the specific products on a CEQ8800 genotyping system. For this purpose, specific PCR runs for labelling and amplification of specific products were performed under specific conditions described in section 2.6.1.

Sets of primers with polymorphism and co-dominance of the markers were demonstrated using ordinary tilapia broodstock genomic DNA (genotypic XY male and XX female from different families).

Briefly, six non-related adult males and four non-related adult females along with a total of 18 offspring (originating from different crosses involving the mentioned adults) were tested for polymorphism and co-dominance. After PCR amplification from genomic DNA with fluorescent primers and running samples on the CEQ genotyping system mentioned before, fragments of different sizes were scored across the different individuals. Furthermore, the offspring were checked for Mendelian segregation of the parental alleles.

4.4 Results

4.4.1 Fragment screening

Constructs were pooled together after standardising the concentrations to 100 ng/µl. As demonstrated in Figure 4.3 (A), using *Sau*3AI restriction enzyme, the pool of plasmids appeared to be digested to completion, resulting in a variety of distinct bands against a background "smear" in the range of 100bp to more than 1000bp. Fragments in the range 200 to 600bp were excised and gel purified as described in section 2.4.2. Gel purified fragments are shown in Figure 4.3 (B). The concentration of the pool of selected fragments was measured by spectrophotometry, to be 36 ng/µl but then diluted with TE buffer down to 10 ng/µl.

Different proportions of vector:insert (3:1, 1:1, 1:3 molar ratio) were initially ligated in small scale test reactions and applied on 27 cm^2 agar plates (Table 4.3 and 4.4). From these tests, the 1:1 molar ratio was selected for full scale preparation and microsatellite screening.

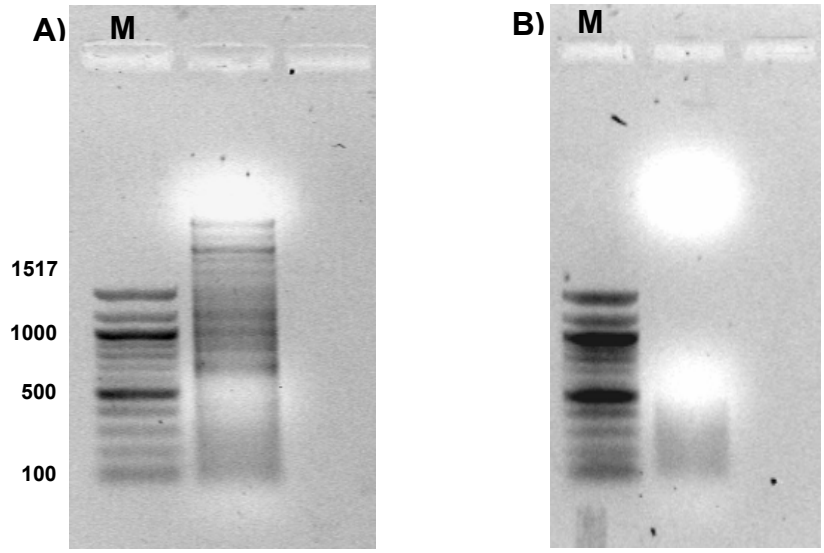

Figure 4.3 A) Digestion of pool of plasmids with *SAU*3AI restriction enzyme and B) Selected fragments (200-600bp) after gel extraction and purification. Marker (M) represents 100bp ladder, indicating the scale in bp on the left of picture A. 1.5% agarose gels.

Vector:insert sizes from white and blue colonies were checked. Running PCR reactions with M13 primers, white colonies gave a range of amplicons as shown in Figure 4.4, from 300bp (lane 3) to nearly 1000bp (lane 4), demonstrating insert sizes range between 0.5-750bp with an average insert size of 300bp. Weak blue colony and strong blue colony bands were observed in the range between 150bp (lane 20) to 450bp (lane 10) with an amplified average vector size of 300bp. Although few variations from the expected sizes were observed, most of the colonies were matching the expectations according to the colour displayed by the colony on X-gal / IPTG LB-agar.

Table 4.3 Master mixes tried during the ligation-transformation optimisation stage. Every master mix from the vector:insert 3:1, 1:1 and 1:3 molar ratios were plated in triplicate, so the observed number of total colonies reflects the average of those three plates. In contrast, negative and positive controls reflect the results of only one plate per master mix.

Master Mixes	Expected	Observed (spreading 150µl of solution)
Negative control 1	Few, vector can't religate after dephosphorylation	2 white / 13 blue colonies
Negative control 2	Few, absence of Ligase	4 white / 2 blue colonies
Positive control 3	All blue colonies due to uncut vector	All blue colonies (90% of surface covered)
Positive control 4	All blue colonies due to vector joining to itself	All blue colonies (75% of surface covered)
Vector/ Insert 3:1	Combined white and blue colonies	> 700 colonies
Vector/ Insert 1:1	Combined white and blue colonies	> 800 colonies
Vector/ Insert 1:3	Combined white and blue colonies	> 500 colonies

Table 4.4 Detailed comparison of the different proportions of vector:insert ligation referring as 3:1, 1:1 and 1:3 molar ratios.

Molar ratio proportions	No. colonies in 100µl	No. colonies in 150µl	% White colonies in 100µl	% White colonies in 150µl	Total colonies in 100µl	Total colonies in 150µl
Vector/ Insert 3:1	266 white/ 220 blue	507 white/ 409 blue	54.7%	55.3%	486	916
Vector/ Insert 1:1	398 white/ 302 blue	654 white/ 457 blue	56.9%	58.9%	700	1,111
Vector/ Insert 1:3	298 white/ 148 blue	409 white/ 114 blue	66.8%	78.2%	446	523

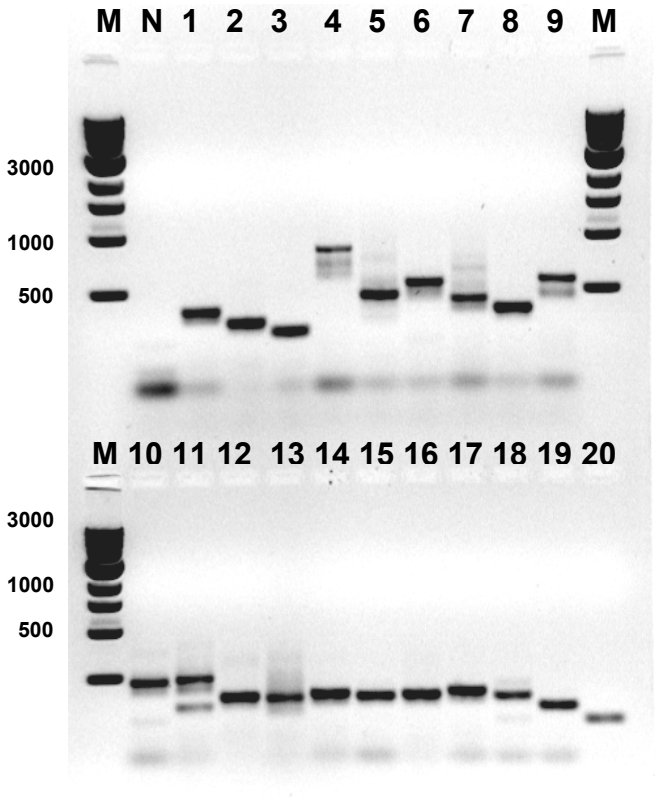

Figure 4.4 PCR amplification of subcloned fragments using M13 primers and selected from LB-agar according to the colony colour. Lines 1 to 9 represent white colonies; lines 10 to 15 represent weak blue colonies; and lines 16 to 20 represent strong blue colonies. The length of pBluescript amplified with no insert showed a band around 300bp. Marker (M) represents 1Kb ladder, indicating the scale in bp at the top-left of the picture and negative control (N) shown after first marker line. 1.5% agarose gel.

In the end, 2,000 colonies were obtained on a large LB-agar plate. From this plate, 29 positive colonies (1.45%) were detected by radiolabelled probes with strong signals, 43 colonies (2.15%) with medium signals and 52 colonies (2.6%) with weak signals. Totals of 124 colonies were re-plated on LB-agar with X-gal and IPTG of which 100 were confirmed as white colonies. These 100 colonies were arrayed in two 96 well plates and screened one last time (following replication onto hybond-N+ membrane) to confirm the strength of the hybridisation signal with the radiolabelled probes. Only 64 positive colonies

were obtained from this screen, and they were selected for sequence analysis. They included 50 colonies (78.0%) with strong and medium signal and only 14 (22.0%) with a weak signal.

4.4.2 Screening of Selected Tilapia BAC clones

The most common tandem repeats observed from sequenced fragments were complete dinucleotide repeats composing both CA and CT motifs.

Microsatellite nomenclature was based on the position of subclones on two original arrayed 96 well plates (e.g. 1A2 refers to plate 1, well A2).

Data from 14 clones that gave clear chromatograms, an identificable microsatellite motif and potential flanking regions suitable for primer design were further analysed. Considering complete sequences (microsatellite motif and flanking regions) for cluster analysis, they resolved into 5 contigs (showing >80% homology) that clustered 15 sequences out of the 22 total sequences analysed (Table 4.5).

Contig 1 was integrated with AF016497 (accession number for *O. niloticus* clone 20-33 microsatellite sequence) and EF438178 (accession number for *O. niloticus* clone OnC4E09-20 repetitive genomic sequence) due to the great overlapping with these sequences observed after blast analysis (see section 4.4.3). It was observed that contig 2 could be integrated into contig 1 if the level of overlapping was relaxed from 80% to a minimum of 60%, being possible meaningful on the way that both contigs mostly overlap well on both complex microsatellite (CA/CT) flanking regions including the repetitive motif but differing

significantly by a 77 bp insertion (contig 2) just after one of the microsatellite flanking regions.

Table 4.5 Contigs created from the comparison of twenty two sequences allowing a minimum of 80% overlapping. Accession number EF438178 refers to clone OnC4E09-20 repetitive genomic sequence and AF016497 refers to clone 20-33 microsatellite sequence, both from *O. niloticus*. Seven sequences were not grouped in any cluster.

Contig no.	Micro. Well ID	Contig length (bp)	Total seq. length (bp)
1	1H5, 1E3, 1H1, 1B9, 1A3, AF016497, EF438178	1158	3378
2 (Contig 1?)	1G7, 1A6	609	963
3	1B3, 1A7, 1E9	749	1076
4	1G6, 1H2, 1D11	448	800
5	1A5, 1E2	558	677
Not clustered sequences	1A1, 1G9, 2H2, 1A11, 1B11, 2G3, 2B4	--	2825

The follow sequence alignment (Figure 4.5) shows seven sequences composing Contig 1; the first two sequences are named according to the accession number for the sequences described above. AF016497 is taken as the reference sequence (609 bp with no overlap were removed at the beginning of this sequence to simplify the view). Regions flanking the polymorphic microsatellite CA/CT are well conserved with some exceptions, showing deletions (e.g. bases 241-244), insertions (e.g. bases 259-260) and single substitutions (e.g. base 385 and 416). These general conserved regions could be considered between the base portions 163 and 590 (427 bp).

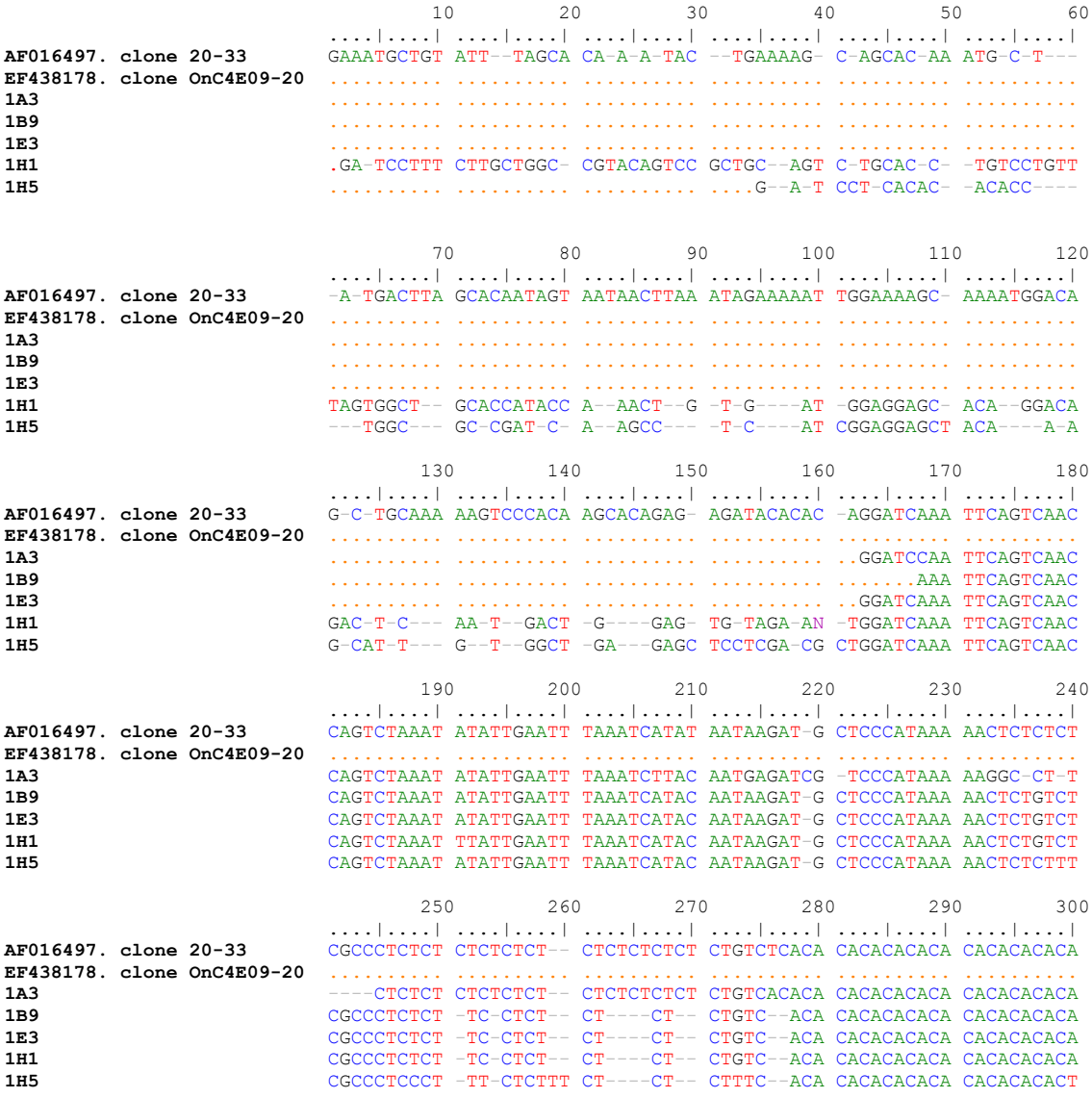

Figure 4.5 Sequence alignment composing Contig 1 with a minimum of 80% of overlapping. The sequence from *O. niloticus* with accession number AF016497 (clone 20-33) is shown first as a base comparison.

```
                                310        320        330        340        350        360
                           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
AF016497. clone 20-33      CACACACACA CACACACACA CACAC-A-C- AG--G-GAGA GAAAA-TCAA GTTTCTAGGT
EF438178. clone OnC4E09-20 .......... .......... .ACAT----- ATTTNCNAGA -A-AAGC-AA GTTTCTAGGT
1A3                        CACACACACA CACACACACA CACAAGATC
1B9                        CACACACACA CACACACACA CACAC----- AG--G-GAGA GAGAAGT-AA GTTTTTAGCT
1E3                        CACACACACA CACACACACA CACAC----- AG--G-GAGA GAGAAGT-AA GTTTCTAGCT
1H1                        CACACACACA CACACACACA CACAC----- AG--G-GAGA GAGAAGT-AA GTTTCTAGCT
1H5                        CACACACACA CACAC----A C--AC----- AG--G-GAGA GA-AAATCAA GTTTCTAGGT

                                370        380        390        400        410        420
                           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
AF016497. clone 20-33      CAGTCAAGCA GCCTGGGAGC TGCTAAATTT GAATCCACCA ATCAGAGAGG CTGTGTACTT
EF438178. clone OnC4E09-20 CAGTCAAGCA GCCTGGGAGC TGCTAAATTT GAATCCACCA ATCAGAGAGG CTGTGTACTT
1A3
1B9                        CAGTCAAGCA GCTTGGGAGC TGCTGAATTT GAATCCACCA ATCAGAGAGG CTGTGCACTT
1E3                        CAGTCAAGCA GCCTGGGAGC TGCTGAATTT GAATCCACCA ATCAGAGAGG CTGTGCACTT
1H1                        CAGTCAAGCA GCCTGGGAGC TGCTGAATTT GAATCCACCA ATCAGAGAGG CTGTGCACTT
1H5                        CAGTCAAGCA GCCTGGGAGC TGCTAAATTT GAATCCACCA ATCAGAGAGG CTGTGTACTT

                                430        440        450        460        470        480
                           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
AF016497. clone 20-33      TTTCCCGCCA AAACAGGTGC AGCCG-TTTT TACACACACA G-AGCACAGA GACAGGATTT
EF438178. clone OnC4E09-20 TTTCCCGCCA AAACAGGTGC AGCCG-TTTT TACACACACA G-AGCACAGA GACAGGATTT
1A3
1B9                        TTTCCCGCCA AAACAGGTGC AGCCG-TTTT TACACACACA G-AGCACAGA GACAGGATTT
1E3                        TTTCCCGCCA AAACAGGTGC AGCCG-TTTT TACACACACA G-AGCACAGA GACAGGATTT
1H1                        TTTCCCGCCA AAACAGGTGC AGCCG-TTTT TACACACACA G-AGCACAGA GACAGGATTT
1H5                        TTTCCCGCCA AAACAAGTGC A-CCTCTTTT TGCACACAC- GCAGCACAGA GACAGGATTT

                                490        500        510        520        530        540
                           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
AF016497. clone 20-33      CTGCAGTGAA TTTCTC-ATA GTGAGGATTC CCCTCAAACA AATGG
EF438178. clone OnC4E09-20 CTGCAGTGTA TTTCTC-ATA GTGAGGATTC CCCTCAAACA AATGACCA-T AATTTCCTAA
1A3
1B9                        CTGCAGTGAA TTTCTC-ATA GTGAGGATTC CTCTCAAACA AATGGCCA-T AACTTCCTAA
1E3                        CTGCAGTGAA TTTCTC-ATA GTGAGGATTC CTCTCAAACA AATGGCCA-T AACTTCCTAA
1H1                        CTGCAGTGAA TTTCTC-ATA GTGAGGATTC CTCTCAAACA AATGGCCA-T AACTTCCTAA
1H5                        GTGCTGTCTA TTT-TCCATA GTGAGGACTC CCCTCAAACA AATGTCTAAT AATTTCCTAA

                                550        560        570        580        590        600
                           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
AF016497. clone 20-33
EF438178. clone OnC4E09-20 CCGTAGGGGC TAGAACGG-T CATTCTTACA
1A3
1B9                        CCGTAGGGGN TAGAACGG-T CATTCTTACA CCGTTTTGTT CAGAAGAGAT C
1E3                        CCGTAGGGGC TAGAACGG-T CATTCTTACA CCGTTTTGTT CAGAAGAGAT C
1H1                        CCGTAGGGGC TAGAACGG-T CATTCTTACA CCGTTTTGTT CAGAAGA-AT C
1H5                        CCGTAGGGGC TAGAACGGGT CATTCTAACA CCGTTTTGTT CAGAAGACAT --GGGGGATCC
```

Figure 4.8 (Continued) Sequence alignment composing Contig 1 with a minimum of 80% of overlapping. The sequence from *O. niloticus* with accession number AF016497 (clone 20-33) is shown first as a base comparison.

Primer sets were tested under standard and optimised PCR conditions with the corresponding subclone, tilapia XY and XX gDNA, tilapia BAC clone containing the brain aromatase gene (as a negative control) and the fifteen original tilapia BAC clones observed to hybridise on choromosome 1 (Table 4.6).

All primer sets amplified products when subcloned plasmid was used as template. However, a product in the expected size range (200-600bp) was observed in 12 out of the 14 primer sets.

Table 4.6 Fourteen primer sets developed from subcloned plasmids that gave successful subclone/microsatellite amplification.

New Marker	5' → 3' Forward and Reverse primers	STS size	PCR Temp.
1A6,F	TCCAGGCTTTTTGACCCACCTA	238bp	60°C
1A6,R	GGATCCAATTCAATCAACCAGTGTAA		
1A7,F	CGCCACCATTAAGAGCAAAAA	143bp	60°C
1A7,R	GAAGCGGGTCAAAGAAGC		
1A11,F	ATGGGCCTGCCGCTGATGCTA	196bp	60°C
1A11,R	AGGACCTGAAGTGGGAGACC		
1B9,F	GTGTAAAAACGGCTGCACCT	197bp	60°C
1B9,R	GTCTCGCCCTCTCTTCCTCT		
1B11,F	ATTGCGCCGACGGGGTGAGG	159bp	60°C
1B11,R	CATGTTTGTGTATTGGCAGGTGTG		
1D11,F	TCCCATAAAAAGGCTTTCTCTCT	100bp	57°C
1D11,R	TTTAGACTGGTTGACTGAATTGGA		
1E3,F	GTGTAAAAACGGCTGCACCT	197bp	60°C
1E3,R	AGAGGAAGAGAGGGCGAGAC		
1E9,F	CCCATAAAAAGGCTTTCTCTCTC	202bp	57°C
1E9,R	GAAAAGTGAAGCAGCGAAGG		
1G6,F	AGACTGGTTGACTGAATTGGA	137bp	60°C
1G6,R	TGATCCCAAGAAGAGTTGCTG		
1H1,F	CAAACTGTGATGGAGGAGCA	243bp	60°C
1H1,R	CAGGCTGCTTGACTGAGCTA		
1H2,F	CGATGAGATGCTCCCATAAAA	223bp	60°C
1H2,R	CAACTGCTTTCTCCCCAGAG		
1H5,F	TTTGGCGGGAAAAAGTACAC	248bp	60°C
1H5,R	CGCTGGATCAAATTCAGTCA		
2G3,F	TCCAATTCAGTCACCAATGC	233bp	57°C
2G3,R	AGGCTTTCCCCGGTATTTTA		
2H2,F	GGATCATCCCATGAAAAGG	230bp	60°C
2H2,R	GACGTCATTTACCGGGACAG		

Successful amplification was much more limited with BAC or genomic DNA template. Thus only 1A6, 1B9, 1B11, 1H5 and 1D11 showed amplification from XX and XY genomic DNA (Figure 4.6, 4.7 and 4.8).

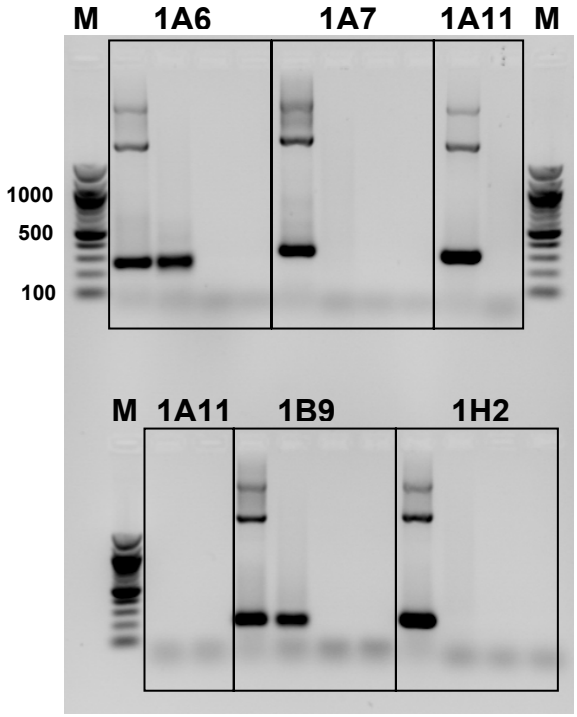

Figure 4.6 PCR amplification of the 1A6, 1A7, 1A11, 1B9 and 1H2 microsatellite sequences using an annealing temperature of 60°C. Each set of primers shows four lanes; the first lane represents the respective subclone (with the sequenced insert); the second lane a male tilapia XY gDNA; the third lane the BAC clone containing brain aromatase gene (as a negative control); and the fourth lane the negative control with no template. Marker (M) represents 100bp ladder, indicating the scale at the top-left of picture. Only 1A6 and 1B9 amplified a product from gDNA. 1.5% agarose gel.

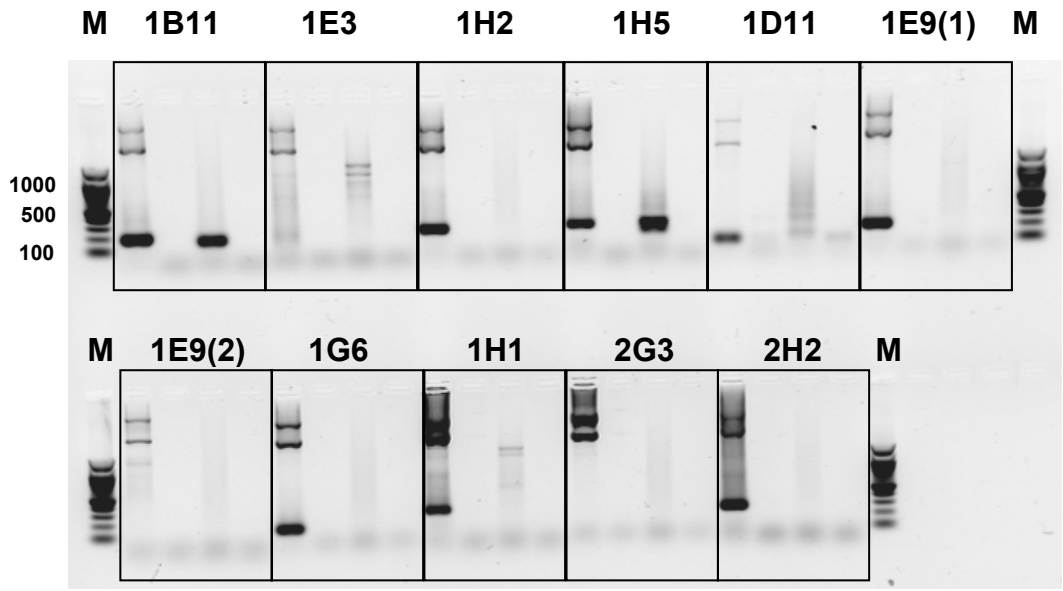

Figure 4.7 PCR amplification of the 1B11, 1E3, 1H2, 1H5, 1D11, 1E9, 1E9, 1G6, 1H1, 2G3, 2H2 microsatellite sequences using an annealing temperature of 60°C. Each set of primers shows four lanes; the first lane represents the respective subclone (with the sequenced insert of about 200 bp); the second lane the BAC clone containing the brain aromatase gene (as a negative control); the third lane a male tilapia XY gDNA; and the fourth lane the negative control with no template. Only 1B11, 1H5 and possible 1D11 amplified a fragment from the subclone and gDNA. 1E9(1) and 1E9(2) refer to the same forward sequence but with two different sets of primers used. Marker (M) represents 100bp ladder, indicating the scale at the top-left of picture. 1.5% agarose gel.

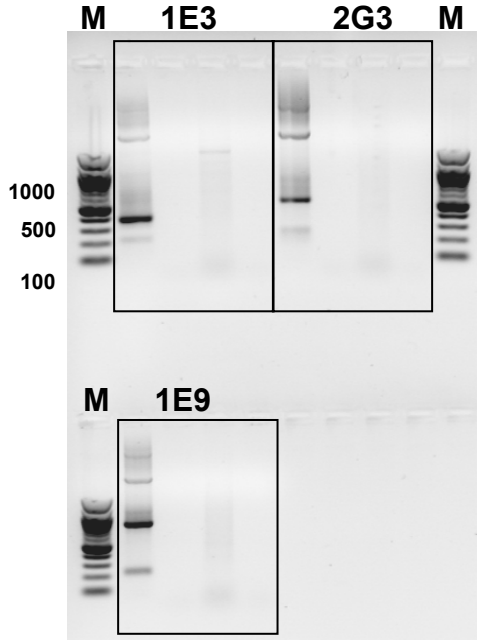

Figure 4.8 PCR optimisations of the 1E3, 2G3 and 1E9 microsatellite sequences using annealing temperature of 57°C. Each set of primers shows four lanes; the first lane represents the respective subclone (with the sequenced insert); the second lane the BAC clone containing brain aromatase gene (as a negative control); the third lane a male tilapia XY gDNA; the fourth lane the negative control with no template. PCR products although were obtained from subclones after PCR optimisation, gDNA still did not amplify. Marker (M) represents 100bp ladder, indicating the scale at the top-left of picture. 1.5% agarose gel.

Eight primer sets (the above five sets that worked for genomic DNA plus three others as negative controls, 1E9, 1H1 and 2H2) were taken forward and tested on the original 15 BAC clones. The three potential negative controls gave no specific products. The five “test” primers gave two distinct patterns of results.

First, four sets (1A6, 1B9, 1H5 and 1D11) gave amplification in multiple clones (Figure 4.9). They many known to be characterised for containing SINE sequences, as previously demonstrated (Figure 4.5) for the majority of the BAC clones included in the present study. As the BACs were originally selected from different regions of chromosome 1, it was considered unlikely that any of these primer sets would identify locus specific regions. Second, only one primer set

1B11 gave a PCR product of an expected size in a single BAC clone (Figure 4.9), considered then as a specific marker. A summary of the positive and negative PCR amplifications from the 14 primer sets analysed is given in Table 4.7.

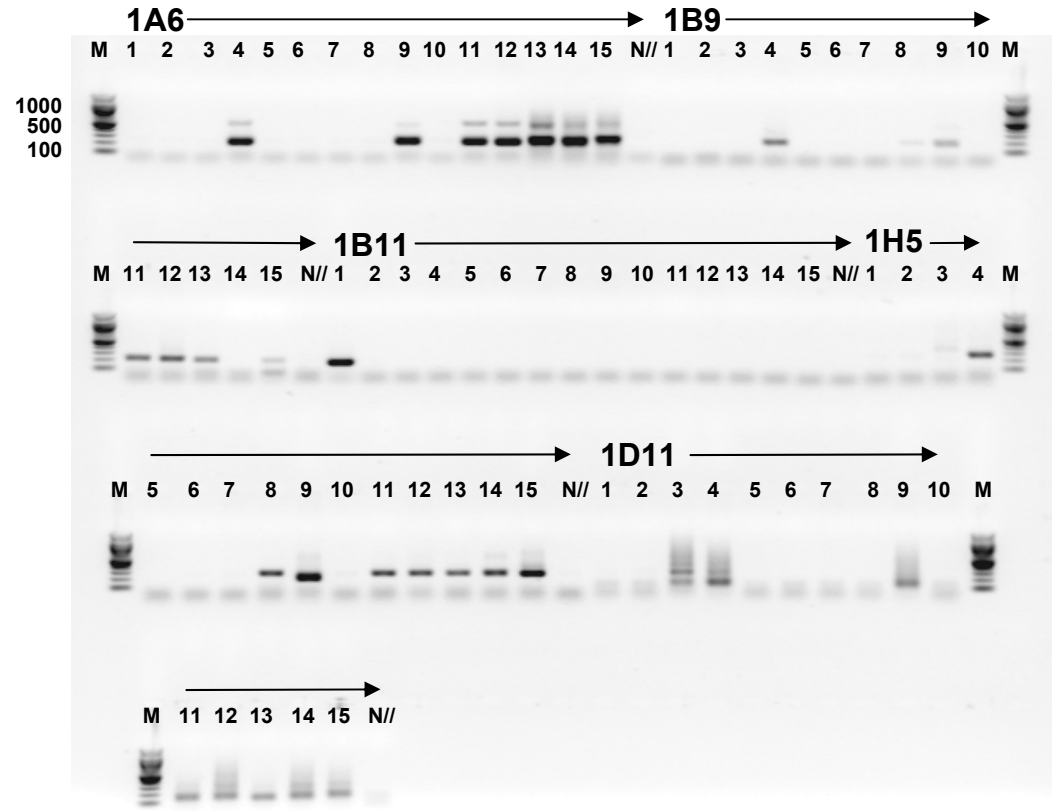

Figure 4.9 Screening by PCR of the original 15 BAC clones selected from chromosome 1 using 60°C annealing temperature. Microsatellite sequences amplified were 1A6, 1B9, 1B11, 1H5 and 1D11. See Table 4.1 for the entire BAC clone references. Marker (M) represents 100bp ladder, indicating the scale at the top-left of picture. Negative control is indicated by an N. 1.5% agarose gel.

4.4.3 Validation of the new microsatellite and tilapia broodstock screening

From the list of potential and already designed microsatellites, only the microsatellite *Oni*159 (previously named 1B11 and coming from the section

above) was taken for further analysis to confirm polymorphism, co-dominance and informativeness.

Table 4.7 Summary of PCR amplifications observed from the new microsatellite sequences, showing positive (+) and negative (-) amplification. Original BAC clones used for screening are indicated by numbers from 1 to 15 (see Table 4.1 for the entire BAC clone references).

Micro. Well ID	Subcloned fragment	Brain aromatase clone	XY gDNA	XX gDNA	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1A6	+	--	+	+	--	--	--	+	--	--	--	--	+	--	+	+	+	+	+
1A7	+	--	--	--															
1A11	+	--	--	--															
1B9	+	--	+	+	--	--	--	+	--	--	--	+	+	--	+	+	+	--	+
1B11	+	--	+	+	+	--	--	--	--	--	--	--	--	--	--	--	--	--	--
1D11	+	--	+	+	--	--	+	+	--	--	--	--	+	--	+	+	+	+	+
1E3	+?	--	--	--															
1E9	+	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
1G6	+	--	--	--															
1H1	+	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
1H2	+	--	--	--															
1H5	+	--	+	+	--	--	--	+	--	--	--	+	+	--	+	+	+	+	+
2G3	+?	--	--	--															
2H2	+	--	--	--	+	+	+	+	+	+	+	+	+	+	+	--	+	+	--

The full process and results are described in depth on Chapter 5. Briefly, *Oni*159 was demonstrated to be polymorphic (testing F2s from three family crosses) generating allele sizes of 167, 175 and 189 bp. It also showed

informativeness in two of the three crosses. Finally, in 8 out of 10 broodstock analysed, this marker was demonstrated to be heterozygous.

4.4.4 BLAST screening

Additional sequence analysis was carried with the 64 sequences from the subcloned colonies.

In 10 of the 12 cases, BLASTn (e-value < e^{-10}) identified sequence homologies to *Astatotilapia burtoni* clone BAC 20D21 (accession number DQ386647). Analysis output of this sequence by Tandem Repeat Finder (TRF) is shown in Table 4.8. *Astatotilapia burtoni* sequence revealed to contain highly repetitive/complex sequences, other than the identified dinucleotide microsatellite which was the focus of the current study. Besides, in 6 of the 12 cases, Nile tilapia clone 20-33 microsatellite sequence (accession number AF016497) was the second most common homology sequence. *Astatotilapia burtoni* and Nile tilapia popularity relates to the fact that sequences 1A3, 1A6, 1B9, 1H1 and 1H5 showed relevant similarity amongst them (as described in the previous section) and having in common a complex microsatellite CA/CT (but with different lengths) which seems to be quite abundant on chromosome 1, and one or two common flanking region of about 81 bp.

Table 4.8 Output from the Tandem Repeat Finder (TRF) program for *Astatotilapia burtoni* clone BAC 20D21 microsatellite sequence (accession number DQ386647). This shows the sequence to comprise nine different repeat sequences of various motif sizes (ranging from 2 to 112 bp), indicators of a highly repetitive DNA sequence.

Position Range (bp)	Repeat Size	Copy Number	Consensus Size	Percent Matches	Percent Indels
165--302	66	2.1	66	98	0
382--448	19	3.6	18	77	4
421--611	56	3.4	55	86	2
421--555	19	7.2	19	70	12
509--593	19	4.5	19	72	8
494--647	56	2.8	56	87	5
432--647	112	1.9	112	80	5
798--840	2	21.5	2	87	0
840--891	2	26.0	2	100	0

Microsatellites that were successfully amplified from previous section (4.4.2) were named properly according to the initials of the species and the length of the amplified sequence. These are *Oni*238, *Oni*197, *Oni*159, *Oni*248, and *Oni*100 standing for the original well IDs 1A6, 1B9, 1B11, 1H5 and 1D11 respectively. *Oni*238, *Oni*197 & *Oni*248 sequences showed more than 197bp identity similarities to the Nile Tilapia *Oreochromis niloticus* Clone 22-33 (accession number: AF016497), and together with *Oni*100 showed closely related flanking regions but with different tandem repeat sizes. Despite differences observed within these sequences, cluster analysis demonstrated that *Oni*197 and *Oni*248 belong to the same cluster, with 80% minimum alignment as mentioned in the beginning of the present section. *Oni*159 showed 426bp identity to the *A. burtoni* clone BAC 20D21 (accession number: DQ386647), and finally *Oni*100 showed 75bp identity to the same previous

sequence after blast analysis but covering a different region (see Table 4.9). A detailed sequence description of these five markers is given in Table 4.10.

Table 4.9 Microsatellite markers generated after the screening of 15 BAC clones from a tilapia BAC library. The % of coverage refers to the microsatellite marker designed.

MARKER NAME	FORWARD 5'-3'	REVERSE 5'-3'	PCR PRODUCT SIZE	TANDEM REPEAT	BAC clone amplified	BLAST Similarities (% coverage)	E-value
*Oni*238 (1A6)	GGATCCAATTC AATCAACCAGT GTAA	TCCAGGCTTTTT GACCCACCTA	238 bp	$(CA)_{25}$ / $(CT)_{13}$	SINE1,2,3, 4,6, 7, 8	Nile Tilapia Clone 20-33 (51%)	5e-41
*Oni*197 (1B9)	GTCTCGCCCTC TCTTCCTCT	GTGTAAAAACG GCTGCACCT	197 bp	$(CA)_{25}$ / $(CT)_{10}$	SINE1,2,3, 4,6, 7, 8	Nile Tilapia Clone 20-33 (78%)	4e-78
*Oni*159 (1B11)	GGATCTGGGTC TGAGTGACC	CTGAAGGTGCC TGTCACGA	159bp	$(CA)_{13}$	DMRT 1	*A. burtoni* clone BAC 20D21 (20%)	8e-04
*Oni*248 (1H5)	CGCTGGATCAA ATTCAGTCA	TTTGGCGGGAA AAAGTACAC	248bp	$(CA)_{22}$	SINE1,2,3, 4,6, 7, 8	Nile Tilapia Clone 20-33 (66%)	2e-65
*Oni*100 (1D11)	TTTAGACTGGTT GACTGAATTGG A	TCCCATAAAAA GGCTTTCTCTC T	100bp	$(CA)_{11}$ / $(CT)_{14}$	DMRT 4, SINE1,2,3, 4,6,7,8	*A. burtoni* clone BAC 20D21 (54%)	6e-16

Table 4.10 Microsatellite sequences from five successfully amplified markers. In bold is shown the position of primers and the name in brackets represents the well ID used after the identification of positive clones by radiolabelled probes.

MARKER NAME	CONSENSUS SEQUENCE
***Oni*238 (1A6)**	5'**GATCCAATTCAATCAACCAGTGTAA**ATATATTGATTTTAAATCACACAATAAG AAACACCCATAAAAAGGCTCTCTCTCTCTCTCTCTCTGTCAATACACACACACAC ACACACACACACAGACACACACACACACACACACACACACACACGCAGCAGCAG CAGAAGCAAGTGAACAAACAGGGGCTGTACATACAGTGTTTCCTGTATGTTTC**TA GGTGGGTCAAAAAGCCTGGA**GCTGCTTAATTTGAATCCACCAATCAGAAAGGCT ATGTACTTTTTCCCGCCAAAACAGGTGCACCTGTTTTTACACACGCAGCACAGAG ACAGGATTTGTGCAGTCTATTTTTCATAGTGAGGACTCCTCTCAAACAAATGGCTA TAATTTCCTTACCAGCTGCGCCACTGGGAAAGACAGTGAGCTCTTGGGAAACAGG GTGATGAGCAGTCAGAATTAGATC'3
***Oni*1197 (1B9)**	5'AAATTCAGTCAACCAGTCTAAATATATTGAATTTAAATCATACAATAAGATGCTCC CATAAAAACTCT**GTCTCGCCCTCTCTTCCTCT**CTCTCTGTCACACACACACACACA CACACACACACACACACACACACACACACACACACACAGGGAGAGAGAAGTAAGTTTTT AGCTCAGTCAAGCAGCTTGGGAGCTGCTGAATTTGAATCCACCAATCAGAGAGGC TGTGCACTTTTTCCCGCCAAAAC**AGGTGCAGCCGTTTTTACAC**ACACAGAGCACA GAGACAGGATTTCTGCAGTGAATTTCTCATAGTGAGGATTCCTCTCAAACAAATGG CCATAACTTCCTAACCGTAGGGGNTAGAACGGTCATTCTTACACCGTTTTGTTCAG AAGAGATC'3
***Oni*159 (1B11)**	5'**CTGAAGGTGCCTGTCACGA**CGGCACCCACACACACACACACACACACACACACC TGCCAATACACAAACATGGAACGCAGGAACAGCAGCAGTGCAGAACACACACATAT AATAAGTCCACAACTGTTCAGGGACTCTA**GGTCACTCAGACCCAGATC**'3
***Oni*248 (1H5)**	5'**CGCTGGATCAAATTCAGTCA**ACCAGTCTAAATATATTGAATTTAAATCATACAATA AGATGCTCCCATAAAAACTCTCTTTCGCCCTCCCTTTCTCTTTCTCTCTTTCACACAC ACACACACACACACACTCACACACACACACACACACAGGGAGAGAAAATCAAGTTT CTAGGTCAGTCAAGCAGCCTGGGAGCTGCTAAATTTGAATCCACCAATCAGAGAGG CT**GTGTACTTTTTCCCGCCAAA**'3
***Oni*100 (1D11)**	5'**TCCCATAAAAAGGCTTTCTCTCT**CTCTCTCTCTCTCTCTCTCTCTCTGGCACAC ACACACACACACACACAAGG**TCCAATTCAGTCAACCAGTCTAAA**'3

The five sets of primers were designed to run under the same PCR conditions, showing clear amplification under the standard conditions mentioned on Table 2.1 and 2.2 using a concentration of 1.5mM $MgCl_2$ and 30 cycles at 60°C for annealing temperature.

4.5 Discussion

4.5.1 Efficiency of the procedure for the design of microsatellites

Construct DNA from fifteen BAC clones were originally pooled; each of the clones contained an average of 105 Kb, making 1,575 Kb in total to screen. Considering that it is estimated to find at least 4 microsatellite every 20-30 Kb in vertebrates genome (specially in fish) for all possible repeat motifs of 1-6bp (Zane *et al.,* 2002), one could expect to find more than 300 microsatellites present within the 15 clones screened. By using 4 different probe combinations (CA, CT, GACA, GATA) for the screening of microsatellites and that only AC motif (the most abundant) is found in fish every 37±51 Kb (Neff and Gross, 2001) it could be expected to be targeting at least 100 microsatellites. However, it is relevant to consider that these motifs still represents a small subset of potential microsatellites to be found. Moreover GACA and GATA were screened simply because they were available in the laboratory and not necessarily because they were highly represented in the tilapia genome.

From the 2,000 colonies screened, about 100 positive sub-clones (5%) were detected with a potential repetitive motif but only 64 sub-clones (3.2%) were confirmed to have microsatellites. From 13 of them (0.65%), it was possible to design primers but only five (0.25%) amplified PCR products properly from genomic DNA. Many of the “failed” clones were found to have 1) internal *Sau*3AI sites and 2) original bacterial sequences, suggesting chimaeric origins (Carleton *et al.,* 2002; O'Connell and Wright, 1997). In the end, only one (0.05%) was confirmed to be polymorphic and suitable for linkage analysis. This locus specific marker was identified as being in the same BAC clone as the dmrt1 gene (chromosome 1, Flpter= 0.85) (Boonphakdee, 2005). The other four

primer sets showed affinities to BAC clones containing SINE sequences, suggesting that these four microsatellites along with several SINEs are associated at the same time with the heterochromatinisation process observed in the distal part of the q arm of chromosome 1 (Harvey *et al.,* 2003b).

These figures showed that the 3.2% of positive clones (with repetitive motifs) observed were very close to the expected 3.1% of positive clones in fish, and higher than the 1.67% found in mammals following traditional methods (no enrichment for specific repeats) for microsatellite screening (Zane *et al.,* 2002) as was done in the present research. So, the number of positive clones observed from the 15 BACs was as expected, nevertheless, the number of useful microsatellites was very low.

In relation to this low efficiency, Zane *et al.* (2002) mentioned that a large amount of the positive clones have to be discarded (easily in the order of 50%) during the isolation-characterisation process, leading to a low return for such a significant effort. This situation is called an empirical success rate of microsatellite isolation. Similar observations in relation to the low efficiency in getting useful microsatellites were made by other authors, despite using microsatellite enrichment protocols (Carleton *et al.,* 2002; Handley and Perrin, 2006).

The two most likely reasons for the low numbers of useful microsatellites obtained from the whole process are enlisted as follows:

1. The inclusion in the original pool of tilapia BAC clones from a highly repetitive chromosome region that produced very similar copies of the same fragment many times.

2. Small fragments of 200bp or less were also included in the analysis (during fragment purification), giving lower chances of getting useful microsatellites from them. It means that selected fragments were too short to design flanking primers after the removal of vector contamination (pBeloBAC11 and pBluescript II KS-) and gDNA from *Escherichia coli*.

Despite the targeted microsatellites being longer than 18 bp (the longer the greater chances of being polymorphic (Carleton *et al.,* 2002), several full sequences were short (<200 bp), reducing the chances for flanking primer design. It should be noted that during microsatellite screening, traditional methods tend to select long microsatellites due to the increased opportunity for probes to bind to the target site(s) (Neff and Gross, 2001) but do not necessarily produce a centered microsatellite with enough flanking regions for primer design.

It is important to consider that selection of fragments (according to their length) depends on the genome base composition and endonuclease recognition site sequence of the chosen restriction enzyme (Zane *et al.,* 2002). In this research, the enzyme *Sau*3AI could potentially lead to determine an unequal sampling of the selected BAC clones and give priority to certain types of fragments. The work of Refseth *et al.* (1997) using *Sau*3AI is one of the examples showing that using a single restriction enzyme could lead to a non-random screening of microsatellites from the genome. Nevertheless, it is reported that this problem seems to be underestimated as several protocols ignore its implications (Zane *et al.,* 2002).

It is well known that the most abundant repetitive motif found in tilapia genome to date is CA (Kocher *et al.,* 1998), and it is observed to be homogeneously distributed along the genome (Lee *et al.,* 2005; Neff and Gross, 2001). As a result, an observation supporting the idea of unequal sampling is that not only CA but also CT were very often found in complex arrayed microsatellites, increasing the chances from these microsatellite fragments to hybridise two of the four radiolabel probes used, and as a consequence, strengthening the positive signal and the detection of subsequent colonies (about 37 out of 50 sequenced colonies that showed strong and medium signal). On the contrary, from the 64 positive clones detected, only 2-3 sequences with tetranucleotide repeats were observed.

It is clear at this stage that the observed *Oni*238, *Oni*197, *Oni*248 and *Oni*100 primer amplifications from the BAC clones previously identified (Harvey *et al.,* 2003b) are present along with repetitive sequences (SINEs) (Oliveira *et al.,* 2003), showing several copies of the conserved region (contig 1) on different clones mapped mainly in the distal area of the q arm on chromosome 1. SINEs are ultraconserved regions among different species. They are distributed throughout the genome and do not participate in the synthesis of proteins but replicate within the genome (retroposon sequences) (Tamarin, 2002). The abundance of repetitive sequences on chromosome 1 (such as SINEs) may be the main reason for having several duplicated sequences, decreasing the total effective number of new microsatellites expected as was observed in the great white-toothed shrew (Handley and Perrin, 2006) when genome walking on flanking sequences to sex determination locus on the Y chromosome, obtaining a low success in finding unique sequences.

*Oni*159 (1B11) containing a dinucleotide microsatellite, was demonstrated to have all the characteristics of an ideal genetic marker. These include a length of no more than 200bp, highly polymorphic, co-dominant inheritance. Moreover, this polymorphic microsatellite was exclusively found by PCR in the clone containing the gene dmrt1 and now has the potential to confirm the presence of this copy gene in the terminal region of the q arm in tilapia chromosome 1 (Boonphakdee, 2005) by linkage analysis.

4.5.2 Sequences identified after BLAST

An important reason for finding very few informative microsatellites from the BAC clones used, is that they were highly associated to a highly repetitive and complex region in the tilapia genome (middle of the q arm on chromosome 1) as described in the previous section. In this way, BLASTn analysis confirmed these observations with the associated sequences found, most of them with Cichlid sequences.

According to Carleton *et al.* (2002), homologous sequences with e-values $< 1^{-10}$ resulting from BLASTn analysis, represent relevant similarity to the query (sequence under investigation). These hits showed on several occasions to be related with genes, such as the C-type lectin natural killer receptor-like (KLR) locus (accession number AY495714; Kikuno *et al.,* 2004), and the platelet-derived growth factor receptor beta b together with the colony-stimulating factor 1 receptor b genes (accession number DQ386647), both found in cichlids, including Nile tilapia.

As explained before, Contig 1 was the largest one in comparison to the other 5 created; this was basically due to the great affinity with the large sequence “clones 20-33 microsatellite sequence” with accession number AF016497 and a total length of 1078 bp and the full overlapped sequence “clone OnC4E09-20 repetitive genomic sequence” with accession number EF438178 and a total length of 247 bp. The contribution from the new sequences, specially 1B9, 1E3, 1H1 and 1H5, gave a total length of 1158 bp, which makes them suitable for publication on the NCBI database.

Moreover, BLASTn results and the contig 1 analysis also confirmed that the terminal part of the q arm on chromosome 1 (where most of the SINE BAC clones were localised) is rich in A+T content (62.15%). It favoured stronger DAPI binding as described in Chapter 3 during cytogenetic studies. In addition, the G+C content observed (37.85%) in contig 1 could be related with the similar composition found from Ron-1 SINE like sequence as a big cluster in the middle of the long arm on chromosome 1 (Bryden *et al.,* 1998; Oliveira *et al.,* 2003).

Contig analysis revealed also a great overlapping (duplicated sequences with at least 80% overlapping) with a similar repeat motif. Along with the highly repetitive sequences matched by blast (such as Astatotilapia burtoni clone BAC 20D21, Acc. No. DQ386647) with most of the contigs, it is supported the idea of a region with heterochromatin accumulation. This observation has been also previously suggested on chromosome 1 (Harvey *et al.,* 2003b), in addition to the CiLINE enrichment along the terminal two thirds of the long arm on the same chromosome (Oliveira *et al.,* 1999).

Finally, it was observed that none of the blast results identified sequences with transposon elements, CiLINE2 or Ron-1 elements as was found by Harvey *et al.* (2003b), blasting exactly the same clones under "BLASTn" method. Instead, the present blast findings demonstrated great similarities to *O. niloticus* (Acc. No. AF016497 and EF438178) and *A. burtoni* (Acc. No. DQ386647 and BX005437) clones sequences with an e-value lower than 10^{-14} in all the cases.

4.5.3 Conclusions

Five new microsatellites were designed from 15 BAC clones distributed along the proposed sex chromosome in Nile Tilapia, Chromosome 1. Three had great similarity to an *O. niloticus* repetitive sequence with Acc. No. AF016497 and two to an *A. burtoni* platelet-derived growth factor receptor beta b and colony-stimulating factor 1 receptor b genes with Acc. No. DQ386647.

From these five microsatellite markers, only one was taken for further analysis as only this one gave a single amplification from the BAC clone containing a copy of the dmrt1 gene (chromosome 1), confirming polymorphism, co-dominance and informativeness within four tilapia families analysed (see Chapter 5). Moreover, this clone showed poor homology (e-value $8e^{-04}$) with *Astatotilapia burtoni* BAC clone 20D21.

Due to the low success for isolating microsatellites from the plasmid pool of 15 BAC clones, it is suggested that, to produce a random selection of microsatellites, digestion of plasmids with two or more restriction enzymes in separated reactions and then pooling fragments together should be used (Hamilton *et al.,* 1999). Additionally, increasing the range of selected fragments

up to 1000 bp could improved the number of subcloned fragments with an insert large enough to design flanking primers.

Another suggested improvement in microsatellite screening over the traditional isolation technique used during this study can be done with the use of randomly amplified polymorphic DNA (RAPDs) with some modifications, simply by screening microsatellites by means of southern hybridisation of RAPDs profiles with repeat containing probes, followed by the selective cloning of positive bands (Cifarelli *et al.,* 1995; Richardson *et al.,* 1995). Finally, the primer extension enrichment protocol should also be considered as an alternative for improving the traditional methods of microsatellite isolation in tilapia (Carleton *et al.,* 2002; Kocher *et al.,* 1998), although significant levels of redundancy in the BAC clones selected might still affect the efficiency (Zane *et al.,* 2002).

Chapter 5 LINKAGE MAPPING

5.1 Summary

This Chapter presents the findings observed on genetic recombination of linkage group 1 and 3 with specific emphasis on sex determination locus. Comparisons were made among *O. niloticus* individuals and with a combination of two different genotypes (XY, XX) on phenotypic males and females. The location of red skin colour with black blotching locus was also recorded.

Four families were prepared for these purposes (Fam. A, B, C, D), generating XY males and females, XX males and females, YY males and females, and XY males / XX females respectively. Only Fam. A and B were genotyped in full, because 1) target YY fish from Fam. C were not statistically confirmed after progeny-testing and 2) Fam. D was simply a control for families A, B and C when considering the success obtained from Fam. A and B on mapping sex determination and black blotching loci. A total of 13 microsatellites previously linkage mapped on LG1 (small submetacentric chromosome) and LG3 (chromosome 1), 3 Sex-linked AFLP markers (SLAMS) and 1 new microsatellite *Oni*159 (see Chapter 4: associated with a dmrt1 gene copy) were chosen for mapping in each cross. The three SLAMS along with three microsatellites, failed to be mapped during this process.

XX individuals appeared to generate significantly larger maps than XY, at least on LG3 when comparing genotypes, while XY males presented larger maps than XY neofemales (specially demonstrated on LG1) when comparing phenotypes. However both observations are still not conclusive due to the incomplete maps generated. A putative telomeric inversion was observed on

LG3 on the long arm when compared to a previous linkage map from a hybrid tilapia (*O. niloticus* x *O. aureus*). A specific-sex determination locus was identified on LG1 map from a XY neofemale, which was tightly linked to the marker UNH995 (Flpter=0.67), and indicated different levels of sex-association depending on the allelic strength when comparing alleles from the same locus (X chromosome: 185bp, 194bp; Y chromosome: 235bp, 258bp) and their sex ratios.

A recessive black blotching locus was demonstrated to be associated with LG3, located between the markers GM128 (Flpter=0.20) and GM526 (Flpter=not available) with 95bp and 240bp alleles respectively. Finally, the centromere was confirmed to be closely related (Flpter=0.14) to these red skin colour/ black blotching flanking markers, in light of centromere-gene distances previously calculated by other authors and observations in the physical map from Chapter 3.

LG1 has been proved to have a major role in sex-determination in this species, with a dosage-sex determination model proposed based on allelic and chromosome influence, and LG3 represents an autosomal chromosome associated with red skin colour in *O. niloticus*.

5.2 Introduction

5.2.1 Sex determination approach

It is well accepted that sex determination in tilapias is based on major (sex chromosome) genes, which presents as XY and WZ systems in closely related species (Ezaz *et al.,* 2006) but is affected by minor (autosomal) modifiers (Avtalion and Hammerman, 1978; Karayucel *et al.,* 2004; Lee *et al.*, 2004; Mair and Abucay, 2001; Shirak *et al.,* 2002; Wohlfarth and Wedekind, 1991) and environmental conditions such as temperature (Baroiller *et al.,* 1995, 1996).

Sex chromosome differentiation (heteromorphism) in tilapia is not well established karyologically (Harvey *et al.,* 2003b), suggesting a primitive and labile sexuality common to most fishes studied (Purdom, 1993), and compared with other vertebrate groups. Nevertheless, SC studies have revealed subterminal unpairing in the largest bivalent during early pachytene in heterogametic *O. niloticus* (Carrasco *et al.,* 1999), and in the longest and one of the short bivalents (with almost full unpairing in the short bivalent) in heterogametic *O. aureus* (Campos-Ramos *et al.,* 2001). These later observations were supported with an epistatic interaction detected between two putative sex determining loci in *O. aureus* (Lee *et al.,* 2004).

This epistatic interaction could lead to a better understanding of the sex determination pathways; for instance, in mammals, the male dominant gene SRY, evolved from SOX3, acts upstream of DMRT1 (one of the most common genes involved in sex differentiation in vertebrates) in the sex determination process (Morrish and Sinclair, 2002). Its male-dominant action is likely to have been epistatic to DMRT1 to produce XYZZ males and XXZZ females,

suggested that this transition happened when sex chromosomes were minimally differentiated (Ezaz *et al.,* 2006). Following the same example, despite the presence of a dominant SRY gene, the over- or under- expression of the combined genes involved in testis development (dosage-dependent), in addition to mutations, in mammals can lead to sex reversal (Mittwoch, 2006).

In fish, even in gonochoristic species, the dosage-dependent effect has been observed spontaneously in different species with a polygenic sex determination and genotype-environment interaction (Ezaz *et al.,* 2006), as observed in some members of the family Cichlidae (*Oreochromis* spp. mentioned above) and Poecilidae (Purdom, 1993).

In tilapia, more than one autosomal locus has influence on sex-reversal. The partial penetrance of unlinked autosomal loci in *O. niloticus* causes sex reversal in both directions (Ezaz *et al.,* 2004c) and these seem to be related with genetic intrapopulation variation. Nevertheless, it had been demonstrated that it is possible to “purge” these autosomal sex-modifying factors within two generations (selecting YY male breeders with the best performance to cross with XX females and viceversa), increasing the mean proportion of males by 6% (Mair and Abucay, 2001). These findings demonstrated also that a single major sex determination gene in tilapia was still in place.

5.2.2 Red skin colour

Ornamental fish were the first species where a connection between sex-linked inheritance and colour patterns was observed. Small bright coloured male and a big grey-green coloured female were typical traits for sexual

selection (Purdom, 1993), representing the major reasons for the collection, domestication and study of these species. A clear example is the medaka (*Oryzias latipes*) where the first Y-linked inheritance of a pigment in fish has been demonstrated (Kondo *et al.,* 2001) and enforced with the identification of a Y specific gene dmy/dmrt1bY located on linkage group 9 (Matsuda *et al.,* 2002). A Y-linked body colour gene has also been reported along with other genes that affect the shape of fins in guppies (Volff and Schartl, 2001).

In platyfish (*Xiphophorus maculatus*), a more complex sex determination mechanism has been shown, proposed to be X, Y, W, where males can be either XY or YY and females XX, XW or YW. Tightly linked polymorphic pigment genes have been found on the X and Y chromosome but were absent from the W chromosome (Kallman *et al.,* 1973; Volff and Schartl, 2001).

To date, Lee *et al.* (2005) have made in tilapia (*O.niloticus* x *O. aureus* hybrid) the only report of genetic markers on LG3 associated with the red skin colouration (showing closer relation to the gene Trp1) and where the main sex determining gene in *O. aureus* is located (Lee *et al.,* 2004).

In *O. niloticus*, the red skin colour has been shown to be a dominant characteristic (RR homozygous, and in low proportion Rr heterozygous) with a recessive wild skin colour (rr homozygous) and the presence of black blotches observed in Rr heterozygotes (McAndrew *et al.,* 1988). Karayucel *et al.* (2004) suggested the linkage of an autosomal sex reversal locus to the red colour locus in this species due to deviation from the all-female sex ratio expected in meiotic gynogenetic family. Ezaz (2002), showed that there was a linkage between at least one putative autosomal sex determining locus and the red

body colouration also in meiotic gynogenetic male and female crosses. However, red body colour is not a natural characteristic in wild tilapia populations, which may only indicate that colour traits are not under directional sexual selection by female mate choice in tilapia as contrarily observed in some other African cichlids (Cichlid Genome Consortium, 2005).

Considering previous statements, in the present research, secondary efforts were directed to the localisation of the red skin colour locus based on the segregation of black blotches and to study its possible penetrance in sex determination. Skin colour of broodstock on fish family design was considered for that purpose.

5.2.3 Fish family design

There are different ways of designing fish families for specific genetic mapping purposes. In tilapia species, families have been developed for a range of studies. For example 1) to compare male and female maps from several non-related families in *O. niloticus* (Lee *et al.,* 2003) or from a single family in *O. aureus* (Lee *et al.,* 2004), 2) to map gene-centromere distances for six allozyme loci, sex and red skin colour loci using six meiotic gynogenetic families from *O.niloticus* (diploid progeny produced by suppression of the second meiotic division) (Hussain *et al.,* 1994), 3) to construct the first tilapia genetic map (Kocher *et al.,* 1998) using haploid gynogenetic embryos from *O. niloticus*, or 4) to create an artificial centre of origin (ACO) for comparative genomics F2 hybrid crosses using *O. aureus* x *O. niloticus* (Lee *et al.,* 2005) or any other

commercially important *Oreochromis* species crossed with *Sarotherodon galilaeus* (Agresti *et al.,* 2000).

Variations in recombination rate in different genomic regions is a common feature between males and females. Some of them are due to specific recombination restrictions related to the sex determining genes (Lorch, 2005) on sex chromosomes. Others relate to restrictions on autosomal chromosomes such as human chromosomes where females have 1.65 times larger linkage map than males with a general tendency for having higher crossover rate near centromeres in females and higher near telomeres in males (also associated with small hotspot clusters with recombination rates as high as 140 cM/Mb) despite the fact that both sexes are highly correlated (Nachman, 2002).

To detect sex differences in recombination in linkage maps, one method is to produce two sets of backcross offspring generated from an F1 generation (e.g. crossing two different inbred lines and backcrossing both males and females; Lorch, 2005). Inbred backcross parents result in a homozygous genotype at almost all loci, then any recombination will be detectable from the F1 parent (male or female, separated).

Tilapia provide an advantageous fish model for the study of sex-specific recombinations due to the flexibility of gamete manipulation. The ability to sex reverse individuals (Bromage and Roberts, 1995) is particularly advantageous in allowing the study of one sexual genotype as both sexual phenotypes (e.g. XY male and females).

In the present study, four pedigrees were set up in *O. niloticus*, combining different genotypes (XX, XY and YY) and phenotypes (male and female) in

order to identify any differences in the segregation of sex or red colour loci and characterise the location and distribution of markers with the construction of linkage maps from specific groups. To make possible the comparison from the different target animals on the several family crosses and clearly detect differences in recombination (Sarder *et al.,* 1999), F1 generations were crossed with a gynogenetic clonal line (XX homozygous, which had the same founder origin), making constant any genetic noise the clones could add at the moment of the parental genotyping.

5.2.4 Linkage groups to map

One of the main purposes of the construction of a genetic linkage map in aquaculture is the association of specific markers to the quantitative characteristics (Quantitative Trait Loci, QTL) such as growth, disease resistance, flesh colour or some other desirable trait through marker assisted selection (MAS) (Liu and Cordes, 2004a).

In aquatic species, medium to fine density framework linkage groups have been created for zebrafish (Shimoda *et al.,* 1999), rainbow trout (Sakamoto *et al.,* 2000), brown trout (Gharbi *et al.,* 2006), Chinook salmon (Stein *et al.,* 2001), channel catfish (Waldbieser *et al.,* 2001), tilapia (Lee *et al.,* 2005), pufferfish (Kai *et al.,* 2005), medaka (Kimura *et al.,* 2005), gilthead seabream (Senger *et al.,* 2006), barramundi (Wang *et al.,* 2007), abalone (Sekino and Hara, 2007), oyster (Hubert and Hedgecock, 2004) and shrimp (Wilson *et al.,* 2002) being among the most relevant. Illustrating the great information that could be generated from the study of the genetic maps, basic information has

been used for the localisation of centromeres and the identification of chromosomal regions containing a high degree of segregation distortion in brown trout and rainbow trout (Danzmann *et al.,* 2005; Gharbi *et al.,* 2006), and detection of differences in recombination between sexes in tilapia (Lee *et al.,* 2003; Lee *et al.,* 2004).

Differences in recombination rates during meiosis between sexes are generally reflected by differences in linkage maps (Lorch, 2005). Recombination could bring together alleles on one chromosome with positive effects on fitness, or could bring together deleterious alleles, allowing them to be more efficiently eliminated by selection, such that the balance of their final effect, acts against recombination rates (Lorch, 2005) It has been demonstrated that genetic conflicts should be relevant in the evolution of sex mechanisms in gonochoristic species (Werren and Beukeboom, 1998).

Low frequencies of recombination between sex-linked loci and the sex-determining region have been clearly described in several fish species such platyfish and medaka (Purdom, 1993), catfish (Waldbieser *et al.,* 2001) and salmonids (Woram *et al.,* 2003). It has been observed that in most fish studied, lower recombination rates are showed in males than females. Most of these fish species have an XY system (Danzmann and Gharbi, 2001). However, the relative influence of physiological effects, as observed in male medaka along the sex chromosome (Matsuda *et al.,* 1999), and susceptibility to environmental effects, observed in many fish species (Strussman and Patino, 1995), that also seem to alter sex-specific recombination rates are largely unknown.

Previous linkage map findings in *Oreochromis* species (mentioned in section 5.2.1) demonstrate that chromosome 1, associated to LG3 (Chapter 3), has to be one of the main LG targets for sex linkage in *Oreochromis* genus, supported at the same time with the observations from the F2 hybrid map (Lee *et al.,* 2005) where only markers from LG3 were significantly associated with the putative sex determination locus. On the other hand, differences in crossover frequencies on LG1 were observed when comparing *O. niloticus* male and females (Lee *et al.,* 2003). Finally, Cnaani *et al.* (2004) and Shirak *et al.* (2006) found marker association with stress response, body colour and sex determination on LG1, 3 and 23, agreeing with previous authors in the location of these QTLs in tilapia. LG23 in particular was observed to be closely linked with sex determination loci and deleterious alleles (Shirak *et al.,* 2002).

In this research, it was considered to carry linkage map studies were carried out only with markers from LG1 and LG3, as physical map observations (Chapter 3) associated with these groups were already available for comparisons and frequent marker association to putative sex loci had been observed on LG1 and LG3 in *O. niloticus* and *O. aureus* respectively.

5.2.5 Selection of genetic markers to map

The information on the location of markers in relation to their proximity to functional genes helps to interpret possible associations between allelic and phenotypic variation in any fish studied (Danzmann and Gharbi, 2001). In this way, linkage groups with well distributed markers and with a high marker density of 2-3cM (Lee *et al.,* 2005) should be relatively precise and useful in

detecting allele-phenotype associations and narrowing the search for target genes as was demonstrated in medaka during the process of finding the Y-specific sex determination gene, DMY (Matsuda *et al.,* 2002).

Type II markers such as AFLPs and microsatellites are some of the most common markers used in the construction of genetic maps with high resolution. They are considered to be non-coding and therefore generally selectively neutral They also have provided to be useful in aquaculture genetics for breeding studies, and more recently as markers linked to QTL (Liu and Cordes, 2004a). Using well characterised families, it is sometimes possible to score the co-dominance from AFLPs (Liu and Cordes, 2004a), and with a relative small size and well conserved flanking sequences, microsatellite genotyping facilitates the comparison and reproducibility of linkage groups from close related species or different strains (Naruse *et al.,* 2000; Gharbi *et al.,* 2006; Matsuda *et al.,* 1999).

Having LG1 and LG3 from *O. niloticus* and *O. aureus* as the main candidates to contain the major putative sex determination loci, several microsatellite markers were selected from the second generation F2 hybrid linkage map published by Lee *et al.* (2005) which were fairly evenly distributed along these two groups. Some of these markers are also reported on studies using exclusively *O. niloticus* (Lee *et al.*, 2003, 2004). In the end, these selected markers served as the basis for the creation of specific linkage maps for the study of recombination differences and localisation of sex determining locus and for the evaluation of the relevance of the red skin colour on sex on target F1 fish with different combination in their genotype and phenotype.

5.2.6 Objectives

- To map putative sex-linked groups in the four Nile tilapia families created, combining genetic markers previously linked by other authors and physical markers associated to chromosome 1 (LG3) and to the small submetacentric chromosome (LG1).
- To investigate the genetic location of the major sex determination gene and the putative sex-influenced red skin colour loci.
- To confirm the orientation of centromeres into the linkage groups using the recombination rates from different families and to find any influence in recombination from phenotypes or genotypes.

5.3 Materials and Methods

In order to explore sex determination in Nile tilapia by linkage mapping, three experiment families (A, B, C) and a control family (D) were bred. The experimental families involved parents with different genotypic and phenotypic sex combinations (see Figure 5.1, 5.2, 5.3 and 5.4). Target animals (F1s) were crossed with XX neomales or females from clonal line A #117 to generate F2s. Offspring were genotyped along with their parents and grandparents to follow allelic segregation. Fin-clipping, tagging and progeny-testing were performed on their corresponding generation, and where available (such as Family B and D), the recording of absence and presence of black blotches.

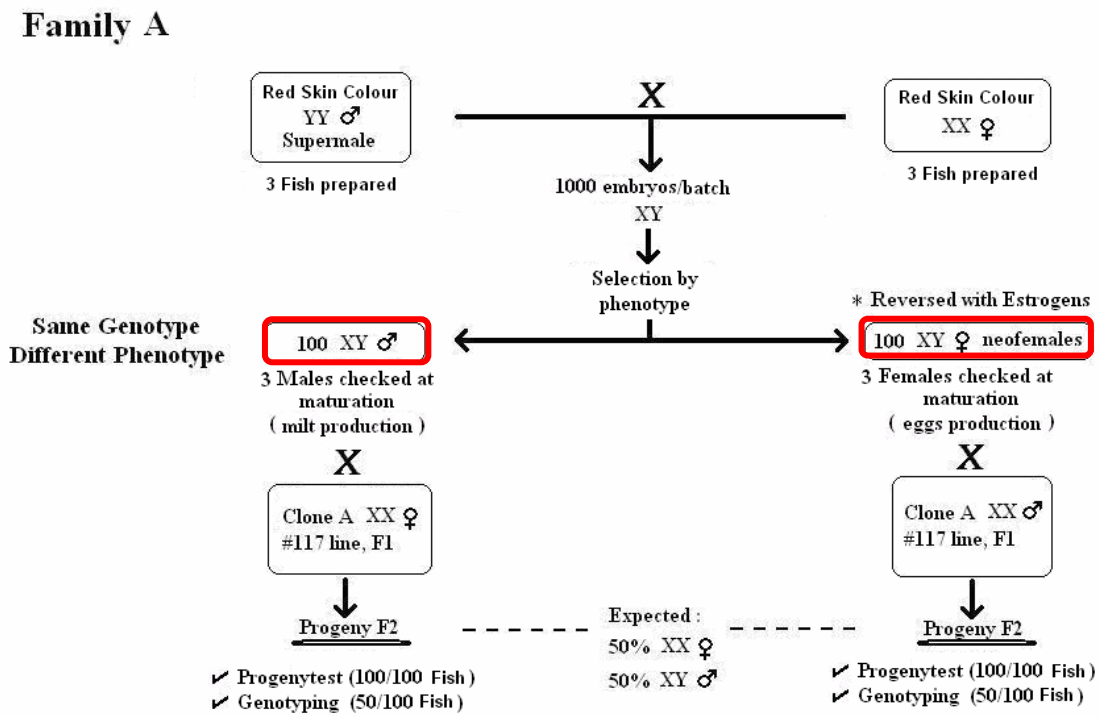

Figure 5.1 Breeding plan from family A showing the target animals (XY males and neofemales) in red boxes.

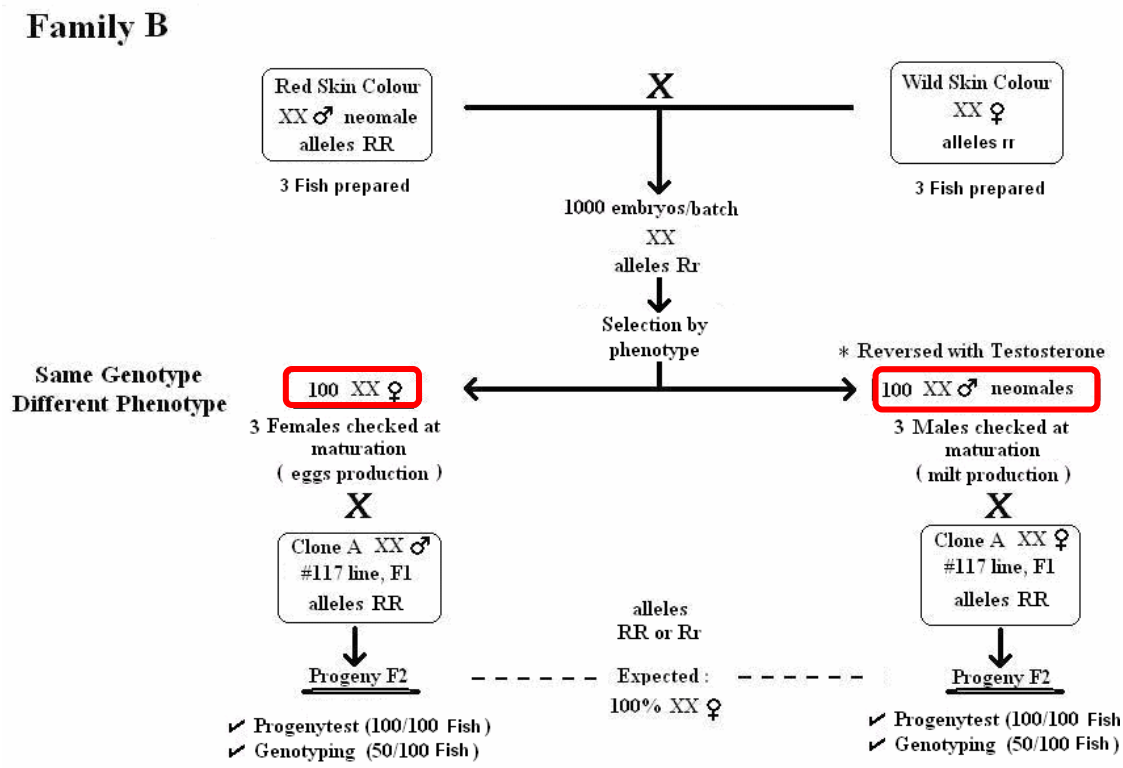

Figure 5.2 Breeding plan from family B showing the target animals (XX neomales and females) in red boxes.

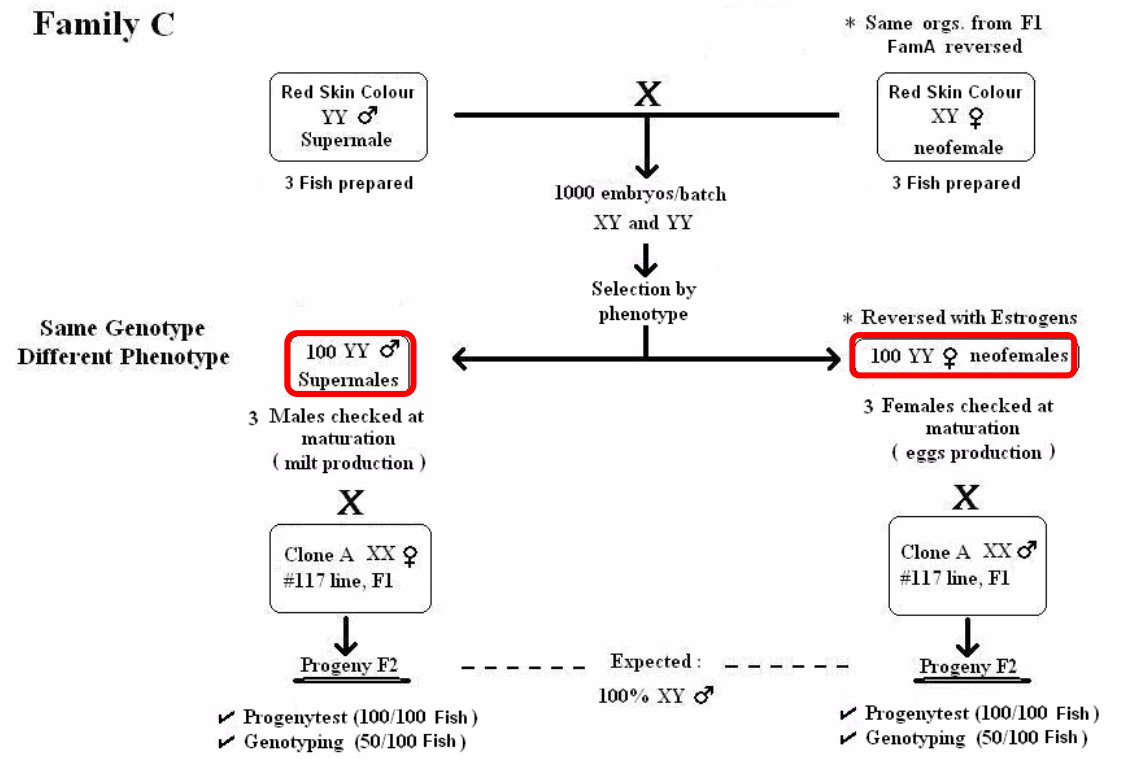

Figure 5.3 Breeding plan from family C showing the target animals (YY male and neofemale) in red boxes.

The linkage mapping process began with the selection of suitable markers. The selection included one new microsatellite marker developed in this research (described in Chapter 4) on chromosome 1 (*Oni*159) 5 microsatellite markers previously linked on LG1 (Lee *et al.*, 2005), 8 microsatellite markers previously linked to LG3 (Lee *et al.*, 2005) and 3 Sex linked AFLPs (SLAMs) identified by Ezaz *et al.* (2004b) on Chromosome 1 in Nile tilapia.

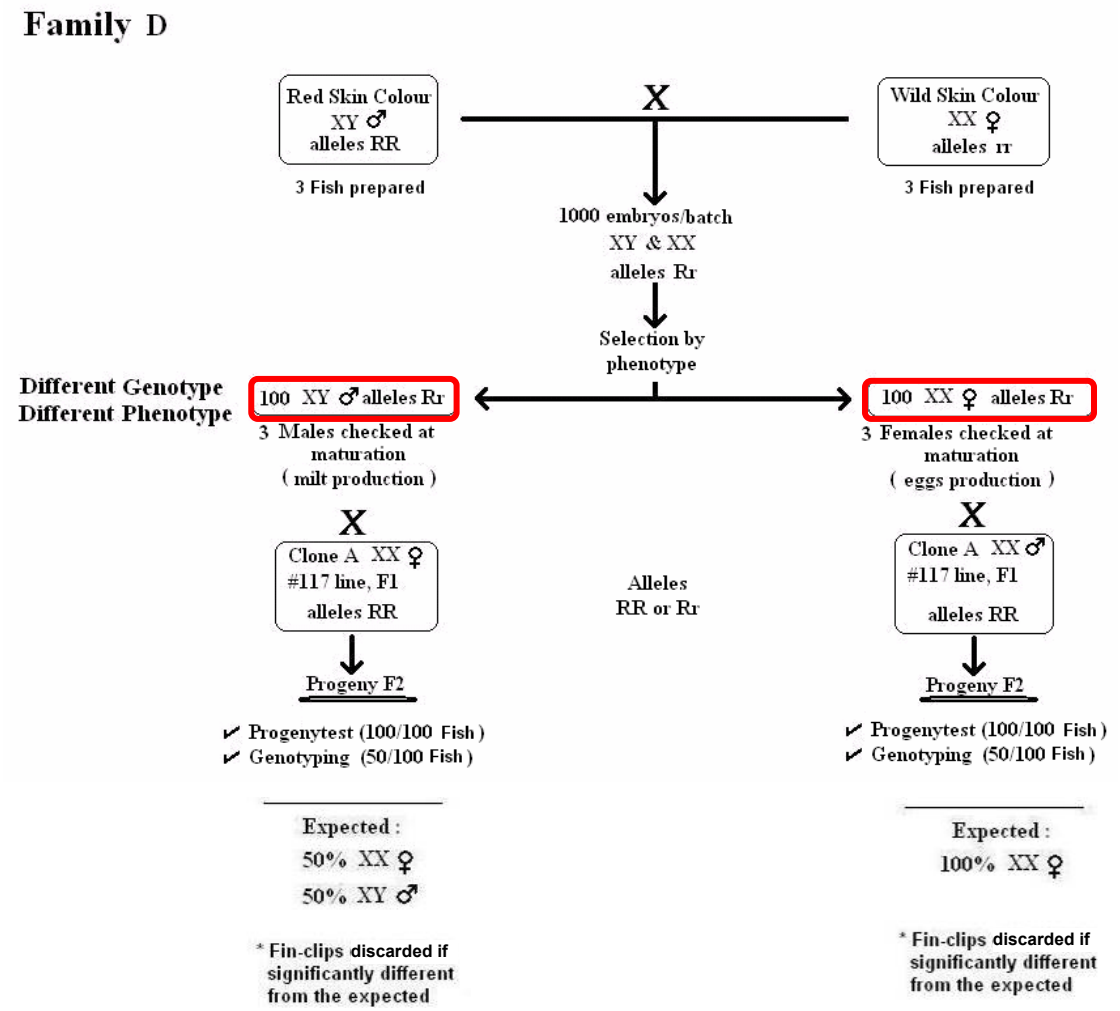

Figure 5.4 Breeding plan from family D showing the target animals (XY male and XX female) in red boxes.

Informativeness of all the markers to map on the specific fish families was checked (parental genotyping). Full genotyping was carried on F2 generations. Genotypes were scored and then analysed using the software LINKMFEX ver.2.3 for the construction of independent linkage groups for each target parent (F1s) and finally, a comparison analysis was carried out with physical maps from Chapter 3, and LG1 and LG3 linkage maps from the present chapter and others previously developed (Lee and Kocher, 2007; Lee *et al.,* 2005), with emphasis firstly on sex determination and secondly on red skin colour with black blotching loci (see Figure 5.5).

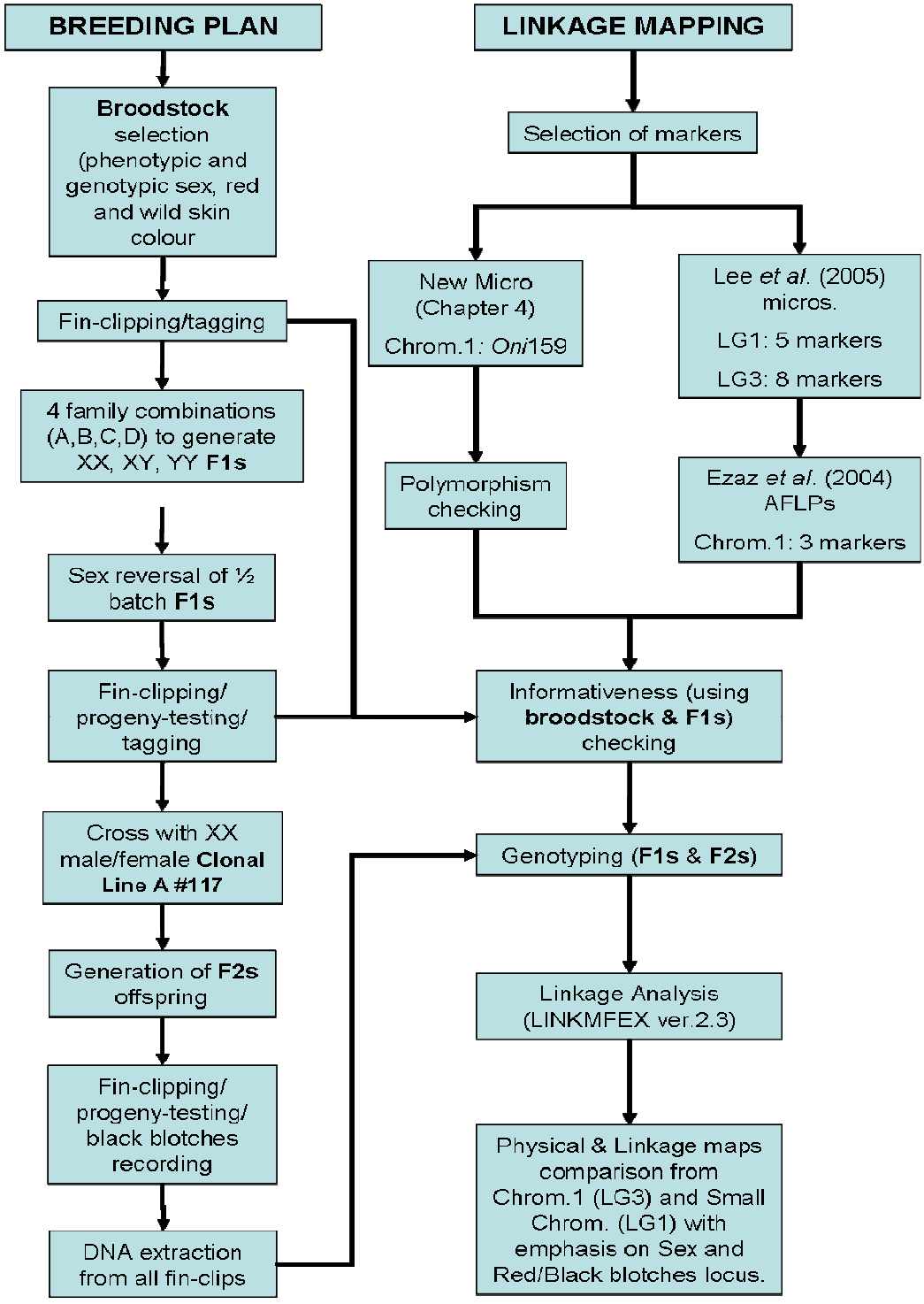

Figure 5.5 Flow diagrams describing the main steps followed for the development of putative linkage maps associated with sex and black blotches in Nile tilapia *O. niloticus*.

5.3.1 Selection of suitable broodstock

The starting mature broodstock from *O. niloticus* var. Stirling were selected from the stock held at the University of Stirling. While the main emphasis was on exploring sex segregation, additional putative genotypes (previously identified by the broodstock's holders staff) were also selected, such as red skin colour XX females, XY males, YY supermales, sex reversed XX neomale and wild skin colour XX females. Physical sex was confirmed by gentle stripping of some milt or eggs and later confirmed by progeny-testing (microscopic examination of gonads). Moreover, some of the sex reversed XY neofemales generated from the cross of a YY supermale and a XX female (F1, Fam A) were used as broodstock for Fam C (Figure 5.3) after progeny-testing too.

Secondarily, only families with a red skin colour male crossed with a wild skin colour female (Fam B and D, see Figure 5.9) broodstock, segregated for absence or presence of black blotches on the skin in a Mendelian expectation.

5.3.2 Description of fish family crosses

In order to analyse the linkage of sex against different phenotypes and genotypes as described above, four fish families (A, B, C and D) were created in triplicate, e.g. three batches of eggs fertilised over a number of weeks, to generate F1s and then F2s. Family A (for comparison of XY male and XY neofemale against sex locus) was initiated with three YY supermales x three XX females, with the expectation of a male proportion close to 100%. Following progeny-testing of 50 F1 individuals per batch (which confirmed the sex ratio

expected), half of each batch was sex reversed into females. Three XY males and three neofemales from the same batch were then crossed with XX female and neomale phenotypes from the clonal line A #117 respectively. F2 generation from both types of crosses were progeny-tested (expecting 1:1 XX/XY sex ratio) and finally genotyped (see Figure 5.1).

Family B (for comparison of XX neomale and XX female mainly against sex locus and secondly black blotch locus) was initiated with crosses from three red skin colour XX neomale x wild skin colour XX female expecting 100% F1 females, checked by progeny-testing. Half of each batch was sex reversed into males and three XX neomales and females from the same batch were crossed with XX females and neomales from the clonal line A #117 respectively. F2 generations from both crosses were progeny-tested (expecting 100% XX females) and genotyped (see Figure 5.2).

Family C (for comparison of YY supermale and YY neofemale against sex locus) was initiated with three YY supermales crossed with three XY neofemales expecting an F1 male proportion close to 100% checked by progeny-testing. Half of each batch was sex reversed into females; then, to increase the chances of finding the right genotype, five putative YY supermales and five putative YY neofemales were then crossed with XX females and neomales from the clonal line A #117 respectively. F2 generations from both crosses were progeny-tested (expecting an XY male rate close to 100%). Once YY identity was confirmed for F1s (by sex ratio), F2 progeny were genotyped (see Figure 5.3).

Family D ("control", comparison of XY male and XX female mainly against sex locus and secondly black blotches locus) was initiated with three red skin colour XY males crossed with three wild skin colour XX females expecting an F1 1:1 sex ratio, checked by progeny-testing. Once they reached maturation, putative XY males and XX females were crossed with XX females and neomales from the clonal line A #117 respectively. F2 generations from both crosses were progeny-tested (expecting 1:1 sex ratio from an F1 XY male and 100% XX females from an F1 XX female); Once F1 identities were confirmed, progeny was genotyped (see Figure 5.4). It is relevant to clarify that family D was created with the purpose to generate natural XY male or XX female (target fish with no sex reversions) from a single cross as controls, and compare them with target fish from family A (XY males and females) and B (XX males and females).

Expected allele combinations for dominant (R) / recessive (r) black blotches are properly described in family A and family D (Figure 5.2 and 5.4 respectively).

The nomenclature used in the present chapter to refer F1 generations is composed by the letter that identifies the family, then the genotype and the phenotype of the target fish such as FamA XYmale, referring to the family A, F1 generation, XY male target animal crossed with a clone XX female to produce an F2.

5.3.3 Sex reversal of F1 generations

Feminisation procedure was applied to XY and YY males for the generation of neofemales, by the administration of diethylstilbestrol (DES) hormone in the food during the first month of external food intake supplied *ad libitum* three times daily (see section 2.2.3). Masculinisation on XX females for the generation of neomales was carried out with 17α-methyltestosterone (MT) hormone (details of feminisation and masculinisation are described in section 2.2.4). 100 to 150 fingerlings were treated per tank and per batch.

5.3.4 Fish quality control and progeny-testing

An evaluation of batch development took place before the actual progeny-testing, quantifying the total number of: eggs spawned, pigmented eggs, embryos and fry. F1 densities were kept at 6 fish/l during the first three months and then reduced to 2 fish/l for up to five months in order to reduce any sex-specific mortality by aggression or increased unequal growth rate. In the case of F2 generations, the whole batches were culled at the time of fin-clipping and progeny-testing.

Three months after hatch, samples of 50 to 100 fish F1 per batch were progeny-tested by gonad squash and acetocarmine treatment (Guerrero and Shelton, 1974) to confirm reversion. Sex ratios were compared with non-reversed fish from the same batch. Once they reached sexual maturation (over five months after hatch, growing at 27°C), 3 to 5 random males or females were selected for crosses with the XX neomale/female clonal line A #117 to generate offspring F2.

These last generations were also progeny-tested as before, culling 50 to 100 fish per batch.

Survival for each batch was recorded at three stages: post-pigmentation (3 days post-fertilisation, dpf), post-hatching (6 dpf) and at yolk sac absorption (12 dpf) (see section 2.2.2). Efficiency of the crosses among the 4 families and within individual families were compared by fitting generalised linear models (GLMs) to the angular percentage of survival relative to the initial number of eggs and significance calculated by one way analysis of variance (ANOVA) using the SPSS v.11 statistical software. For the interpretation of sex ratios, heterogeneity chi-square analysis was performed per individual cross and per family at the F1 and F2 generation level to identify XY, XX and YYs according to the criteria of (Mair *et al.,* 1994). To confirm YY parental status, a statistical cutoff was applied. E.g. for YY × XX genotypes, the offsprings ratio is expected to deviate from a 1:1 sex ratio at a P significant level <0.001. Otherwise the crossed male parent was identified as XY.

5.3.5 Loci used for markers to map

The polymorphic markers used in this analysis are given in Table 5.1. Nine and five microsatellite markers well spaced along LG1 and LG3 markers respectively (Lee and Kocher, 1996; Lee *et al.,* 2005) were selected for genotyping analysis of the four families developed (A, B, C, and D). In addition, the new marker described in Chapter 4 (*Oni*159) and three SLAMs (Ezaz *et al.*, 2004b) were also included in the analysis.

To minimise screening costs, M13 tailing (Raposo, 2001) was employed. A 19bp sequence was appended to the 5' end of one of each primer pair during oligo synthesis (see Table 5.1 and section 2.6.1 for more details). Published primer sequences and Short Tandem Sequences (STS) sizes were obtained from GenBank.

Table 5.1 Markers proposed for linkage analysis, consisting of 1 new microsatellite marker and 8 markers from LG3, 6 markers from LG1 and 3 SLAMs (*OniX*420/*OniY*425 belong to the same locus; Ezaz *et al.*, 2004b).

New Marker	5' → 3' Forward and Reverse primers + M13 tail (lower case)	STS size	PCR Temp.
*Oni*159F	cacgacgttgtaaaacgac CTGAAGGTGCCTGTCACGA	159bp	60°C
*Oni*159R	GGATCTGGGTCTGAGTGACC		
LG3 Marker			
GM354F*	CGGGAGAGCAGGTCAG	117bp	57 °C
GM354R*	gtgctgcaacattttgctg CACGTTCAGGGTTACTGTGTT		
UNH168F	cacgacgttgtaaaacgac TAAGAAGGTTAGAAAGAAAGTG	138bp	57 °C
UNH168R	TATATAATAATTTCCTAAACGGC		
GM271F*	GCAGCTGGATCAGTCTCTG	117bp	57 °C
GM271R*	gtgctgcaacattttgctg TGGGAAGTCGTTCATACAAAG		
UNH115F	cacgacgttgtaaaacgac ACCTTCATCTCGGTCAG	149bp	57°C
UNH115R	TCAAGCAGCTGATTTTT		
GM150F	cacgacgttgtaaaacgac GTCTCAGTTTGTTTGGCTTAC	171bp	57 °C
GM150R	AGGTGATTGGCTTAGATGAT		
GM128F	cacgacgttgtaaaacgac ATGATGAGAGAAAGGGAAAGA	148bp	57 °C
GM128R	CATTACTGTGCCTCTGTGAAG		
GM526F	cacgacgttgtaaaacgac TCTTCCTCAGCCCATCTGTT	221bp	57 °C
GM526R	CAACTGTTGGCAGTGACAGG		
UNH982F	cacgacgttgtaaaacgac TCAATACTGTGGTCCCCTCTTT	133bp	57 °C
UNH982R	TCTCAGAGCGCTATCTTCCTG		

Table 5.1 (Continued) Markers proposed for linkage analysis, consisting of 5 markers from LG1 and 3 SLAMs (*OniX*420/*OniY*425 belong to the same locus; Ezaz *et al.*, 2004b).

LG1 Marker	5' → 3' Forward and Reverse primers + M13 tail (lower case)	STS size	PCR Temp.
GM633F	cacgacgttgtaaaacgac GTGTCCCAAGAAAACCAGGA	188bp	57 °C
GM633R	GACCCAGGACTCATGTGCTT		
UNH995F	cacgacgttgtaaaacgac CCAGCCCTCTGCATAAAGAC	159bp	60°C
UNH995R	GCAGCACAACCACAGTGCTA		
UNH104F*	GCAGTTATTTGTGGTCACTA	138bp	60°C
UNH104R*	gtgctgcaacattttgctg GGTATATGTCTAACTGAAATCC		
GM614F	cacgacgttgtaaaacgac AAGAGGCAGGTTAGCACCAC	110bp	57 °C
GM614R	AGGGAAAGCATTATGCCAAT		
UNH846F	cacgacgttgtaaaacgac TGGAGCAGCTTCTTCTACATCA	229bp	57 °C
UNH846R	CACATGATGGAAGCCGTGTA		
AFLP's markers			
*OniX*420F	cacgacgttgtaaaacgac TTGGCATGTAGATCCGGT	167bp	57 °C
*OniX*420R	TGCTCTTCTGCATACTTTAGC		
*OniY*425F	cacgacgttgtaaaacgac TTGGCATGTAGATCCGGT	193bp	57 °C
*OniY*425R	CTTCTGCATACGGTACTTTAGG		
*OniY*227F	cacgacgttgtaaaacgac CACAGGTATTCATCAGAGCC	169bp	57 °C
*OniY*227R	ACAAACTTCACACAAAACAAA		
*OniY*382F	cacgacgttgtaaaacgac ACTCAAAACTGGTCTCGTGG	286bp	57 °C
*OniY*382R	ACATTTCACAGTGAAACGAGC		

*** = The 19bp M13 tailed primer for GM354, UNH971 and UNH104 markers was wrongly design at the moment of the oligo synthesis by an external company. These errors were noticed after completion of the research; then, special considerations have to be taken during the interpretation of their results.**

5.3.6 DNA extraction and fragment analysis

Finclip DNA extractions were carried using the REAL PUREGENE DNA purification Kit (REAL laboratories, Spain), reducing volumes for 0.2 ml well plate extractions. DNA concentrations were adjusted into a range of 50-100 ng/µl for PCR reactions and checked on an agarose gel for quality/integrity purposes as described in section 2.3.2.

PCR reactions were optimised to get the clearest possible traces during the genotyping analysis (see section 2.6.1). Optimisations were achieved by

modifying the annealing temperature (57°C or 60°C, see Table 5.1), the concentration of $MgCl_2$ (between 1.5-2.0 mM) and the concentration of tailed primer (between 0.2-2.0 pmol). Markers amplified using the standard tailed primer master mix formula (see general materials and methods, Table 2.12) were UNH168, GM271, GM526, UNH982, GM633, UNH995, UNH868, GM614, UNH846, *OniX*420, *OniY*425, *OniY*382, *OniY*227, *Oni*159. For GM354, 2.0 mM of $MgCl_2$ was required; for UNH971, 2.0 mM of $MgCl_2$ and 0.5 pmol tailed primer; for GM150 and GM128, 2.0 pmol of tailed primer.

The 19 markers were tested for informativeness in the broodstock (grandparents) and F1 generation (parents) from the four families. Several animals from the clonal line A #117 were also genotyped to confirm their homozygosity along the whole set of markers. Once suitable markers were identified and allele sizes were recorded, two target fish F1s from each family with the larger number of informative markers were chosen to continue the genotyping of F2s. In other words, for family A, 1 XY male and 1 XY neofemale cross were selected; for family B, 1 XX neomale and 1 XX female cross; for family C, 1 YY male and 1 YY neofemale; for family D, 1 XY male and 1 XY neofemale.

Three fluorescent dyes were used to enable PCR products to be multiplex screened during genotyping: blue (Beckman WellRed dye 4), green (dye 3) and black (dye 2). Blue gave the strongest signal, followed by green and then black. PCR labelled products concentrations needed to be adjusted to get an approximate balanced strength from traces along with the red (dye 1) size standard before genotyping (Figure 5.6).

Most of the markers amplified successfully, and were informative in at least one of the crosses analysed. However, the SLAMS *OniX*420, *OniY*227, *OniY*382 and microsatellite UNH104 were monomorphic in all crosses.

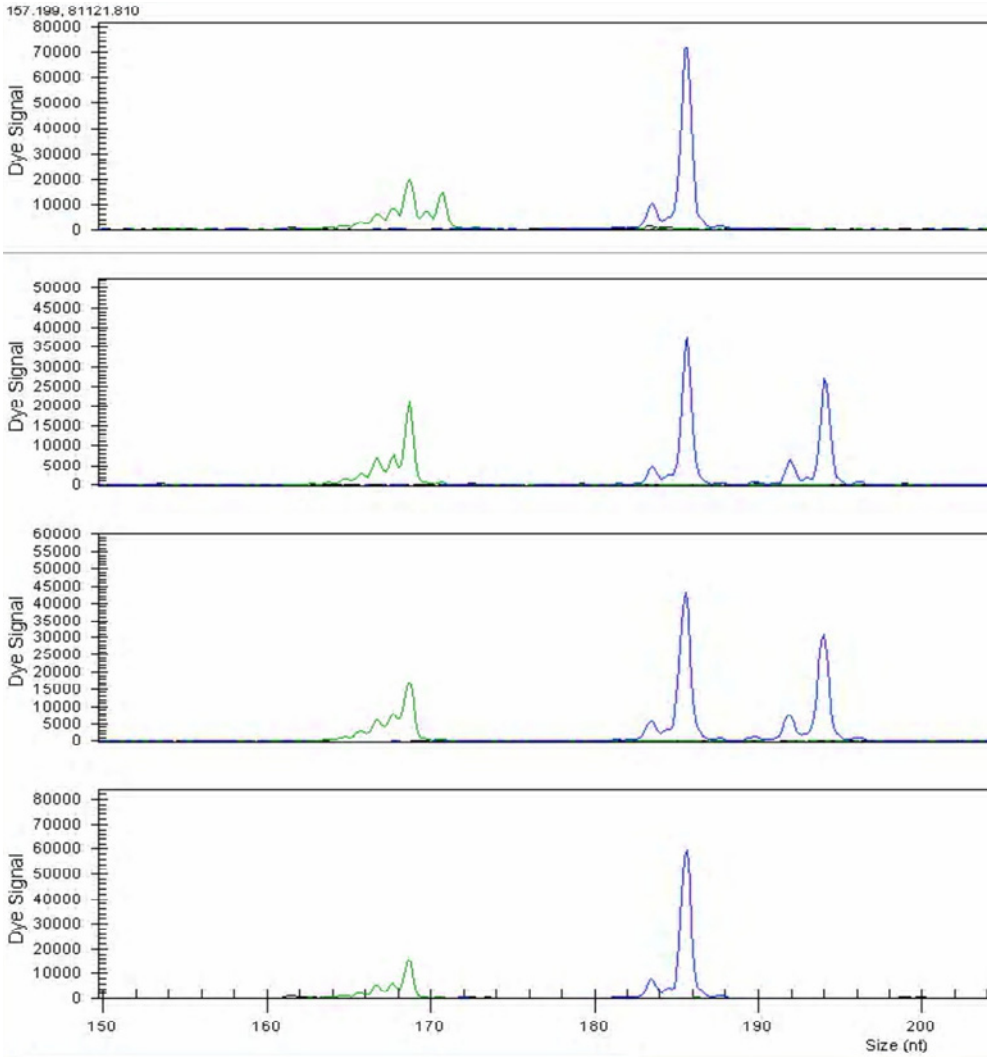

Figure 5.6 Four offspring (F2) from Family A cross with XY neofemale parent showing UNH168 (alleles 168bp and 170bp) and UNH995 (alleles 185bp and 194bp) markers. X axis represents fragment size in bp and Y axis the strength of dye signal.

5.3.7 Linkage analysis and mapping

At this point it is important to clarify that red skin and black blotches were described in this research as if they belong to the same locus due to these two phenotypic characteristics have been observed to segregate together in

families B and D when using wild type females (grandmother) in the starting cross. So the recording of presence or absence of black blotches was used to identify the locus for this putative single gene.

Markers UNH354 and GM271 were difficult to score due to the presence of several variable peaks.

Families C and D were not genotyped at the F2 level. The reasons for this were because family C putative YYs were demonstrated to be not statistically different from a heterogametic (XY) genotype after chi-square analysis of several immature putative YY male and neofemale F2s progeny. Family D was originally developed for alternative comparison studies, primarily investigating sex and secondarily looking at red skin colour segregation, but due to the successful mapping of sex locus and black blotches locus in family A and B, family D was not considered to be crucial for the completion of the present research. Nevertheless it was considered relevant to present the information generated at this point (Broodstock and F1 generation) from family C and D to backup further research on these two families.

The rest of the markers were linked into either LG1 (4 markers) or LG3 (8 markers). Analysing the data with LINKMFEX v. 2.3 software, the significance of recombination between sex and every marker (two-point analyses) was calculated by chi-square analysis under Mendelian expectation for family A, and for family B between black blotches and the markers. For the specific case of family B XX female, the marker UNH995 was two-point compared with the rest of the informative markers on LG1 due to the lack of information on black blotches.

For the construction of linkage groups, a chi-square linkage of markers with significant p-values at 1 degree of freedom were calculated based on recombination frequencies with LINKMFEX v. 2.3 software (downloaded from R.G. Danzman's website: http://www.uoguelph.ca/~rdanzman/software/ LINKMFEX/) using the Theta function (where map distances represent single crossovers between markers) and the sub-components (sub-programmes) LINKMFEX, LNKGRP, MAPORD and MAPDIS during multicomparison analysis. Along with this, Goodness-of-fit (G-test) (Sokal and Rohlf, 1981) were calculated by the same software based on Mendelian expectations from each marker. Using the maximum possible Likelihood of Odds (LOD) scores for each target fish, construction of linkage maps was done by the calculation of recombination rates and statistical significance of the link ($P< 0.05$). Even when some of the markers failed to link all together into the same linkage group for some of the crosses, pairs of linked markers were compared with their putative LG according to Lee *et al.* (2005, 2007) linkage groups. Finally, graphical representations of linkage groups were created with MapChart 2.2 (Voorrips, 2002) (free access software). Linkage groups were named according to the nomenclature described in section 5.3.2 for the F1 target animals.

Sex and black blotches scores (where available) were integrated into the multicomaprison linkage analysis as a single defined locus.

5.3.8 Physical and linkage maps comparisons

Graphical comparisons were carried among previous assigned physical maps (Chapter 3), genetic linkage maps from Lee *et al.* (2005, 2007) and the new genetic maps developed in this chapter (from Family A XY, males and neofemales and family B XX, neomales and females).

5.4 Results

5.4.1 Fish quality control and progeny-testing, statistical indicators

Efficiency of the crosses was evaluated by one way ANOVA for each of the four created families at each of the six monitored developmental stages (total eggs, pigmentation, normal embryos, abnormal embryos, normal fry, abnormal fry). In the F1 generation no significant differences was observed among Families A, B and D, with a mean of total eggs per spawn of 845; 479 pigmented eggs, 456 normal embryos and 432 normal fry, representing an average survival of 51.1% fry just before the first external feeding. The total number of abnormal embryos and fry was relatively low at 1.6% and 0.4% respectively from the total spawned eggs. In contrast, Family C presented significantly lower numbers at F1 ($P< 0.001$) in every stage with respect to families A, B and D, with an average of 227 total eggs, 117 pigmented eggs, 89 normal embryos and 85 normal fry, with an average survival of 30.5%.

In the A, B, C, and D F2 generations, one way ANOVA demonstrated significant differences among the family groups at every stage of development. Total eggs gave an $F=4.77$ ($P< 0.001$), pigmented eggs an $F=2.58$ ($P< 0.05$), normal embryos $F=3.57$ ($P< 0.01$), abnormal embryos $F=4.44$ ($P< 0.001$),

normal fry F=3.35 (P< 0.01) and abnormal fry F=3.19 (P< 0.01). Percentages of survival at every stage revealed differences between males and females within the same family when observing the % of survival, giving evidences of higher survival from crosses made with male target animals than females in all family groups (Table 5.2). Moreover, Fam.C showed the lowest % of survival at every stage of development (except YY males at pigmented eggs stage), mainly reflected on Fam.C YY neofemales.

Progeny-testing was performed for the original broodstock (grandparents) and parents (F1). Analysing the progeny from grandparents by chi-square test, families A, B and C genotypes were correctly confirmed by the significant differences (P< 0.001) observed from 1:1 males/females, while family D showed no significant differences from 1:1 as expected from a normal cross XX x XY female/male (Table 5.3).

Progeny-testing from F1 family A XY males (FFE1, D53A) and neofemales (F11D and F64C) showed significant differences (Table 5.4) with more male progeny in some of the crosses, and no significance in others (EB33, 393D and 52B6) despite the same parent genotypes (XX x XY or XY x XX, female/male respectively). Progeny from family B XX neomales and females were all female. Family C revealed two male F1s (DC44 and CD73) with a P< 0.01 and two neofemale F1s (8A92 and 708B) with P< 0.01 and P< 0.001 respectively (all cases compared to 1:1 sex ratio); according to Mair *et al.* (1994) criteria, only the neofemale 708B (P< 0.001) should be considered with a truly YY genotype.

Interestingly, the cross D529 x 5834 from family C neofemales showed a significant skewed sex ratio (p< 0.001) with more females than males, possibly

referring to the autosomal(s) effect observed by Ezaz *et al.* (2004c) in YY androgenetic families or more specifically by the effect of specific alleles from one of the X chromosomes (in this case coming from XX female 5834) as observed in family A (properly discussed on section 5.5.1).

Progeny from family D XY males gave no significant differences from 1:1 males/females from all the crosses, and XX females gave all females from all crosses (Table 5.4).

Table 5.5 shows the scores observed for the absence and presence of the black blotches from F1 progeny. They were scored only from family B XX neomale and some of the crosses from family D XY male and XX female. All progeny was demonstrated to segregate according to Mendelian expectations except for the progeny that came from two of the crosses from family B XX neomale (EEC3 and 27F8) with $P<0.05$.

Table 5.2 F2 generations early development means from each of the family groups identified by the sexual genotype and phenotype of the target animal. % of survival is based in the initial total eggs.

Stages	F2s Family Groups Means (% survival)							
	A		B		C		D	
	XY MALE	XY NEOFEMALE	XX NEOMALE	XX FEMALE	YY MALE	YY NEOFEMALE	XY MALE	XX FEMALE
N*	3	5	6	4	10	7	5	7
Total Eggs	833	390	646	449	647	332	495	388
Pigmented	420 (50.4%)	120 (30.7%)	347 (53.8%)	289 (64.3%)	246 (38.0%)	81 (24.4%)	317 (64.0%)	167 (43.0%)
Normal Embryos	351 (42.1%)	84 (21.5%)	315 (48.7%)	231 (51.4%)	122 (18.9%)	48 (14.5%)	240 (48.5%)	105 (26.9%)
Abnormal Embryos	32 (3.8%)	9 (2.3%)	9 (1.4%)	18 (4.0%)	7 (1.1%)	6 (1.8%)	22 (4.4%)	6 (1.5%)
Normal Fry	349 (41.9%)	72 (18.5%)	257 (39.8%)	165 (36.7%)	109 (16.8%)	47 (14.2%)	224 (45.2%)	94 (24.2%)
Abnormal Fry	4 (0.5%)	1 (0.3%)	2 (0.3%)	14 (3.1%)	3 (0.4%)	1 (0.3%)	8 (1.6%)	3 (0.7%)

*** = N represents the number of crosses included for the specific analysis of the six development stages.**

Table 5.3 Grandparents progeny-tests and chi-square analysis from family A,B,C and D.

Ref. Broodstock grandparents Female x Male	Grand parents Genotype	Family	Testis	Ovas	Total Progeny Tested	X^2 [1] 1:1	P limits
1268 x 2ED9	XX x YY	A	111	0	111	111.000	<0.001
FA3F x 2ED9	XX x YY	A	42	2	44	36.364	<0.001
F824 x 50BF	XX x XX	B	0	43	43	43.000	<0.001
E766 x 5834	XX x XX	B	0	55	55	55.000	<0.001
F1D6 x F752	XY x YY	C	69	2	71	63.225	<0.001
DF02 x ADB2	XY x YY	C	0	0	0	---	---
F824 x F461	XX x XY	D	175	175	350	0.003	n/s

Table 5.4 Parents progeny-tests (F1) and chi-square analysis from family A, B, C and D. Each family cross is identified by the sexual genotype and phenotype of the target animal. Blue tag denotes target fish showing no significant differences from 1:1 segregation, and red tag denotes target fish with significant differences.

Fam A XY MALE						
Ref. F1 parents Female x Male	**Parents Genotype**	**Testis**	**Ovas**	**Total Progeny**	**X^2 [1] 1:1**	**P limits**
1435 x FFE1	XX x XY	90	22	112	41.286	<0.001
F3EC x EB33	XX x XY	76	84	160	0.400	n/s
FE7A x D53A	XX x XY	56	98	154	11.455	<0.001
TOTAL				**426**		
Fam A XY NEOFEMALE						
Ref. F1 parents Female x Male	**Parents Genotype**	**Testis**	**Ovas**	**Total Progeny**	**X^2 [1] 1:1**	**P limits**
F11D x 50BF	XY x XX	24	8	32	8.000	<0.01
393D x F4F7	XY x XX	26	36	62	1.613	n/s
52B6 x 50BF	XY x XX	12	9	21	0.429	n/s
F64C x F3EE	XY x XX	74	51	125	4.232	<0.05
TOTAL				**240**		
Fam B XX NEOMALE						
Ref. F1 parents Female x Male	**Parents Genotype**	**Testis**	**Ovas**	**Total Progeny**	**X^2 [1] 1:1**	**P limits**
3D70 x 3602	XX x XX	0	48	48	48.000	<0.001
3D70 x 4248	XX x XX	0	150	150	150.000	<0.001
F3EC x EEC3	XX x XX	0	157	157	157.000	<0.001
F3EC x 11F3	XX x XX	0	51	51	51.000	<0.001
E41C x 27F8	XX x XX	0	51	51	51.000	<0.001
E41C x 2FFB	XX x XX	0	50	50	50.000	<0.001
TOTAL				**507**		
Fam B XX FEMALE						
Ref. F1 parents Female x Male	**Parents Genotype**	**Testis**	**Ovas**	**Total Progeny**	**X^2 [1] 1:1**	**P limits**
DD6F x 5834	XX x XX	1	33	34	30.118	<0.001
FCE1 x 50BF	XX x XX	0	50	50	50.000	<0.001
D470 x F4F7	XX x XX	0	50	50	50.000	<0.001
F53F x 5834	XX x XX	0	50	50	50.000	<0.001
TOTAL				**184**		
Fam C YY or XY MALE						
Ref. F1 parents Female x Male	**Parents Genotype**	**Testis**	**Ovas**	**Total Progeny**	**X^2 [1] 1:1**	**P limits**
E41C x F7AA	XX x XY	55	46	101	0.802	n/s
E41C x D729	XX x XY	6	3	9	1.000	n/s
3D70 x DC44	XX x XY	50	25	75	8.333	<0.01
F3EC x CD73	XX x XY	30	10	40	10.000	<0.01
F3EC x D858	XX x XY	10	10	20	0.000	n/s
2785 x D218	XX x XY	3	5	8	0.500	n/s
TOTAL				**265**		

Table 5.4 (Continued) Parents progeny-tests (F1) and chi-square analysis from family A, B, C and D. Each family cross is identified by the sexual genotype and phenotype of the target animal. Blue tag denotes target fish showing no significant differences from 1:1 segregation, and red tag denotes target fish with significant differences.

Fam C YY or XY NEOFEMALE						
Ref. F1 parents Female x Male	**Parents Genotype**	**Testis**	**Ovas**	**Total Progeny**	**X^2 [1] 1:1**	**P limits**
1A5F x 5834	XY x XX	0	5	5	5.000	n/s
D529 x 5822	XY x XX	2	3	5	0.200	n/s
D529 x 5834	XY x XX	13	37	50	11.520	<0.001
8F59 x F3EE	XY x XX	24	26	50	0.080	n/s
8A92 x F4F7	XY x XX	17	4	21	8.048	<0.01
708B x 5834	YY x XX	19	1	20	16.200	<0.001
TOTAL				**151**		
Fam D XY MALE						
Ref. F1 parents Female x Male	**Parents Genotype**	**Testis**	**Ovas**	**Total Progeny**	**X^2 [1] 1:1**	**P limits**
DBFF x 59F6	XX x XY	49	52	101	0.089	n/s
FE7A x E93C	XX x XY	11	15	26	0.615	n/s
D07D x 3A92	XX x XY	74	56	130	2.492	n/s
D07D x D8A9	XX x XY	50	48	98	0.041	n/s
09C8 x FD33	XX x XY	44	56	100	1.440	n/s
TOTAL				**455**		
Fam D XX FEMALE						
Ref. F1 parents Female x Male	**Parents Genotype**	**Testis**	**Ovas**	**Total Progeny**	**X^2 [1] 1:1**	**P limits**
E88F x 5822	XX x XX	0	66	66	66.000	<0.001
E5AB x 5822	XX x XX	0	24	24	24.000	<0.001
0753 x F3EE	XX x XX	0	38	38	38.000	<0.001
E91D x 5834	XX x XX	0	100	100	100.000	<0.001
F9BC x 5834	XX x XX	0	19	19	19.000	<0.001
E5AB x F4F7	XX x XX	0	12	12	12.000	<0.001
E51A x F3EE	XX x XX	0	50	50	50.000	<0.001
TOTAL				**309**		

Table 5.5 Parents black skin blotches scores (F1) and chi-square analysis from family A, B, C and D. Each family cross is identified by the sexual genotype and phenotype of the target animal. Blue tag denotes target fish showing no significant differences from 1:1 segregation, and red tag denotes target fish with significant differences.

Fam B XX NEOMALE						
Ref. F1 parents Female x Male	**Parents Genotype RR x Rr**	**Black Blotches**	**No Black Blotches**	**Total Progeny**	**X^2 [1] 1:1**	**P limits**
3D70 x 3602	XX x XX	24	24	48	0.000	n/s
3D70 x 4248	XX x XX	82	68	150	1.307	n/s
F3EC x EEC3	XX x XX	66	91	157	3.981	<0.05
F3EC x 11F3	XX x XX	27	24	51	0.176	n/s
E41C x 27F8	XX x XX	40	61	101	4.366	<0.05
E41C x 2FFB	XX x XX	30	20	50	2.000	n/s
TOTAL				**557**		
Fam D XY MALE						
Ref. F1 parents Female x Male	**Parents Genotype RR x Rr**	**Black Blotches**	**No Black Blotches**	**Total Progeny**	**X^2 [1] 1:1**	**P limits**
DBFF x 59F6	XX x XY	19	31	50	2.880	n/s
FE7A x E93C	XX x XY	15	11	26	0.615	n/s
TOTAL				**76**		
Fam D XX FEMALE						
Ref. F1 parents Female x Male	**Parents Genotype RR x Rr**	**Black Blotches**	**No Black Blotches**	**Total Progeny**	**X^2 [1] 1:1**	**P limits**
E5AB x 5822	XX x XX	13	11	24	0.167	n/s
0753 x F3EE	XX x XX	24	14	38	2.632	n/s
E91D x 5834	XX x XX	50	50	100	0.000	n/s
E5AB x F4F7	XX x XX	7	5	12	0.333	n/s
E51A x F3EE	XX x XX	53	46	99	0.495	n/s
TOTAL				**273**		

5.4.2 Linkage group 1

Three different versions of linkage group 1 were constructed (Lee *et al.*, 2005, 2007) after the multicomparison analysis of Fam. A and B target animals (Table 5.7). These maps came specifically from Fam.A, XY male and neofemale and Fam. B, XX female. From these two families, only family A was scored for sex as Fam. B consisted of 100% females in F1 progeny; and black

blotches scored only on Fam. B because they were expected to segregate for colour in the F2 progeny.

The informative markers analysed from LG1 were GM633, UNH995, UNH868, GM614 and UNH846.

The two point analyses demonstrated very tight linkage of the sex determining locus to LG1 markers in one (XY neofemale) of the two crosses from Fam.A (Table 5.6). This association was confirmed by chi-square analysis ($P<0.001$) as well as by a high LOD score (13.546). This strong association of sex determination with marker UNH995 was exemplified by all male progeny (24) with the genotype 185/235 and all females progeny (21) with the genotype 185/194. In the other cross involving a Fam.A (XY male), sex was not found to be linked to the marker UNH995 (Table 5.7), with 12 females and 7 males having the genotype 185/258, and 16 females and 9 males being 185/185 genotypes. In this case, chi-square was not significant and the LOD score was very low (0.004).

LOD (likelihood of odds) score is defined as the maximum possible likelihood under the null hypothesis of no segregation (assuming a single normal distribution) (Lynch and Walsh, 1998).

For Fam.B, markers GM633, UNH995 and UNH846 were found to be linked only for the XX female. The same informative loci were not linked in XX neomale from the same family due to a low LOD scores (LOD< 1). Taking UNH995 as a reference during the two point analysis (due to its potential linkage with sex), a closer significant linkage (chi-square= 41.32, $P< 0.001$) and a LOD of 11.12 was observed with the marker UNH846 than GM633 (Table

5.8), corresponding to the closer linkage expected for GM633 from the markers used.

No association of black blotches was observed with LG1 markers from Fam.A and B. Finally, Goodness-of-fit analysis was run for every marker mapped, demonstrating no significant deviation from Mendelian expectations.

Comparisons of the LG1 map from the different crosses against the previous LG1 versions from Lee *et al.* (2005, 2007), markers UNH995 and UNH846 displayed relative consistency but slightly contracted in their distances: 15.2 cM in Fam.A XY male and 12.1 cM in Fam.A XY neofemale and with 19.0 and 14.1 cM in the two previous LG1 maps (Lee *et al.*, 2005, 2007) respectively. Nevertheless, in Fam.B XX female the same markers showed a distance of 41.5 cM.

An inversion of the markers UNH846 and GM614 was observed when comparing Fam.A XY male and neofemale with the LG1 hybrid map presented by Lee *et al.* (2005) (Figure 5.7). This inversion was confirmed when comparing the order of the markers UNH846 and UNH719 from the LG1 maps presented in 2003 and 2007 (Lee *et al.*, 2003, 2007). Fam.B XX female showed an unexpected order for UNH846, locating between GM633 and UNH995 and with a closer distance with the latter (4.0 cM).

The graphical comparison of LG1 from the Fam.A XY neofemale and LG1 Fam.5&7 (Lee and Kocher, 2007) with the physical map from the small chromosome in *O. niloticus* assigned to LG1 gave better evidence of where the sex determining locus may be localised. In Chapter 3, this small chromosome was orientated with the physical localisation of the gene CYP19A1 gene (Flpter

0.37) and UNH995/UNH104 (Flpter 0.67). When comparing LG1 Fam.5&7 and Fam.A XY neofemale, it is evident that the sex determining locus is tightly linked with the marker UNH995 in the new map but also between this marker and the gene WT1b at 34.9 cM in LG1 Fam.5&7 map (Lee and Kocher, 2007). Considering the sum of these findings, it is possible then to suggest a physical localisation of the sex determining locus just before UNH995 (Flpter 0.67) but in a range still not tightly delimited (Figure 5.8). One last observation is the relative similarity in the linkage distance between UNH995 and UNH846 with a previous map, having 14.1 cM in LG1 F5&7 map and 12.1 cM in Fam.A XY neofemale, giving confidence to the observations from the new linkage map developed.

Table 5.6 Fam. A, clonal line A XX neomale crossed with XY neofemale (F3EE x F64C(e)). Informative markers from LG1 (Lee *et al.*, 2005) showing genotypic proportions in males and females of *O. niloticus*. Significance of linkage between sex and markers was tested by chi-square with 1 degree of freedom, and LOD score considering the maximum likelihood.

Marker	SIRE	DAM	PROGENY			X^2 [1] linkage	LOD score (Z_{max})	N
			Genotypes	Female	Male			
GM633	207/207	207/217	207/207	10	18	6.095 **	1.357	42
			207/217	11	3			
UNH995	185/185	194/235	185/235	0	24	45.000 ***	13.546	45
			185/194	21	0			
GM614	113/113	113/117	113/113	1	15	20.512 ***	4.929	41
			113/117	20	5			
UNH846	194/194	192/204	194/194	1	16	23.439 ***	5.739	41
			192/204	20	4			

****P<0.01; ***P<0.001.**

Table 5.7 Fam. A, XY male crossed with clonal line A XX female (D53A x FE7A(c)). Informative markers from LG1 (Lee *et al.*, 2005) showing genotypic proportions of males and females of *O. niloticus*. Significance of linkage between sex and markers was tested by chi-square with 1 degree of freedom, and LOD score considering the maximum likelihood.

Marker	SIRE	DAM	PROGENY			X^2 [1] linkage	LOD score (Z_{max})	N
			Genotypes	Female	Male			
UNH995	185/258	185/185	185/258	12	7	0.021	0.004	47
			185/185	16	9			
GM614	113/117	113/113	113/117	16	8	0.183	0.217	49
			113/113	15	10			
UNH846	192/204	194/194	194/204	14	8	0.020	0.757	49
			192/194	16	11			

Table 5.8 Fam. B, clonal line A XX neomale crossed with XX female (50BF x FCE1(d)). Informative markers from LG1 (Lee *et al.*, 2005) showing genotypic proportions of the marker UNH995 with respect to GM633 and UNH846 in *O. niloticus*. Significance of linkage between UNH995 and the other markers was tested by chi-square with 1 degree of freedom, and LOD score considering the maximum likelihood.

Marker	SIRE	DAM	PROGENY			X^2 [1] linkage	LOD score (Z_{max})	N
			Genotypes	UNH995 (allele 185)	UNH995 (allele 227)			
GM633	206/206	206/218	206/206	15	10	3.000	0.658	48
			206/218	8	15			
UNH995	185/185	185/227	185/185	---	---	---	---	---
			185/227	---	---			
UNH846	194/194	194/198	194/194	22	1	41.326 ***	11.121	49
			194/198	1	25			

***P<0.001.**

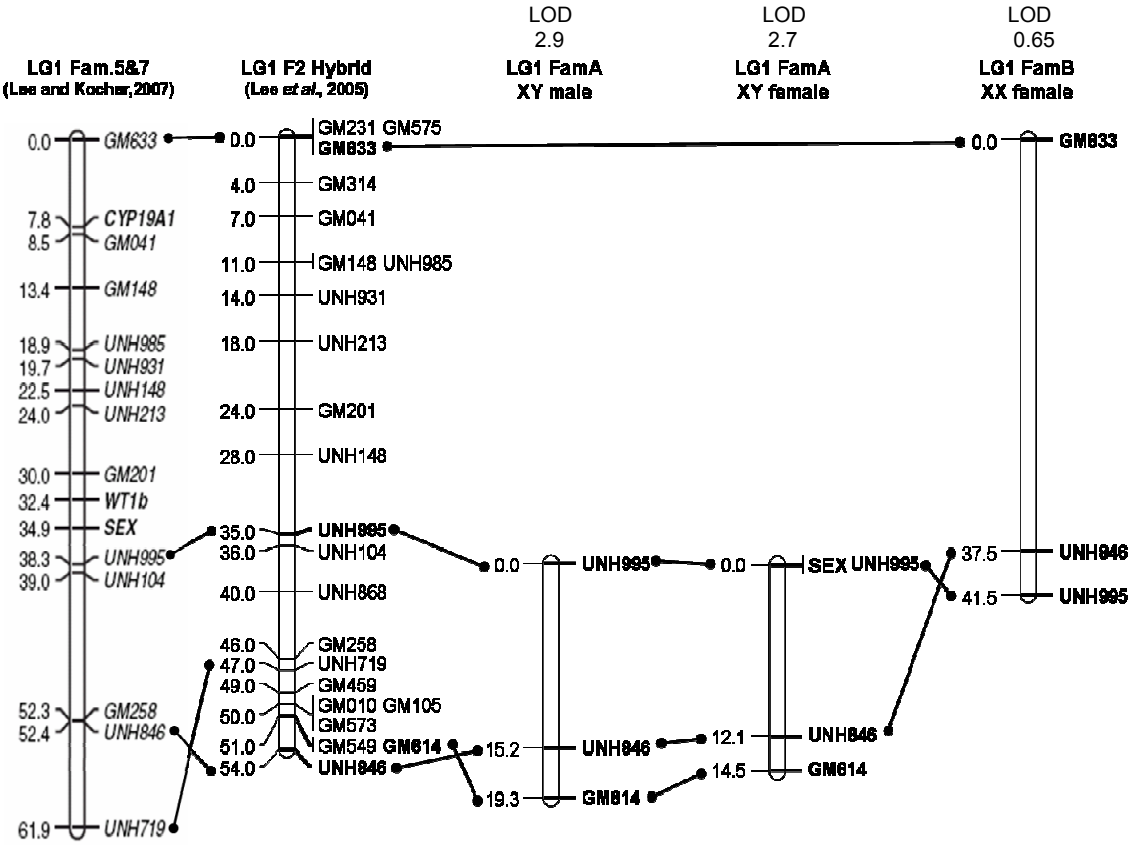

Figure 5.7 Markers from LG1 linked at overall LOD range 0.65-2.9 for the three different linkage groups newly created, and comparing them with LG1 maps developed by Lee *et al.* (2005) from a hybrid cross *O. niloticus* X *O. aureus* and Lee and Kocher (2007) from *O. niloticus*.

5.4.3 Linkage group 3

From the four crosses analysed, it was possible to associate seven markers with LG3 (Lee *et al.,* 2004; Lee *et al.,* 2005) plus the new marker developed in Chapter 4, *Oni*159. In the case of the two crosses from Fam.A, the seven markers were not statistically linked into a single group during the multicomparison analyses but were apparently still in the order expected. According to the information obtained from sex and black blotching loci, results from Fam.A are presented in a two point analysis with sex whilst from Fam.B XX neomale are presented in a two point analysis with black blotches. Results from Fam.B XX female are presented according to the comparison with

UNH168 (a common marker linked in the four crosses analysed) due to the black blotches scores were not recorded for Fam.B XX female progeny.

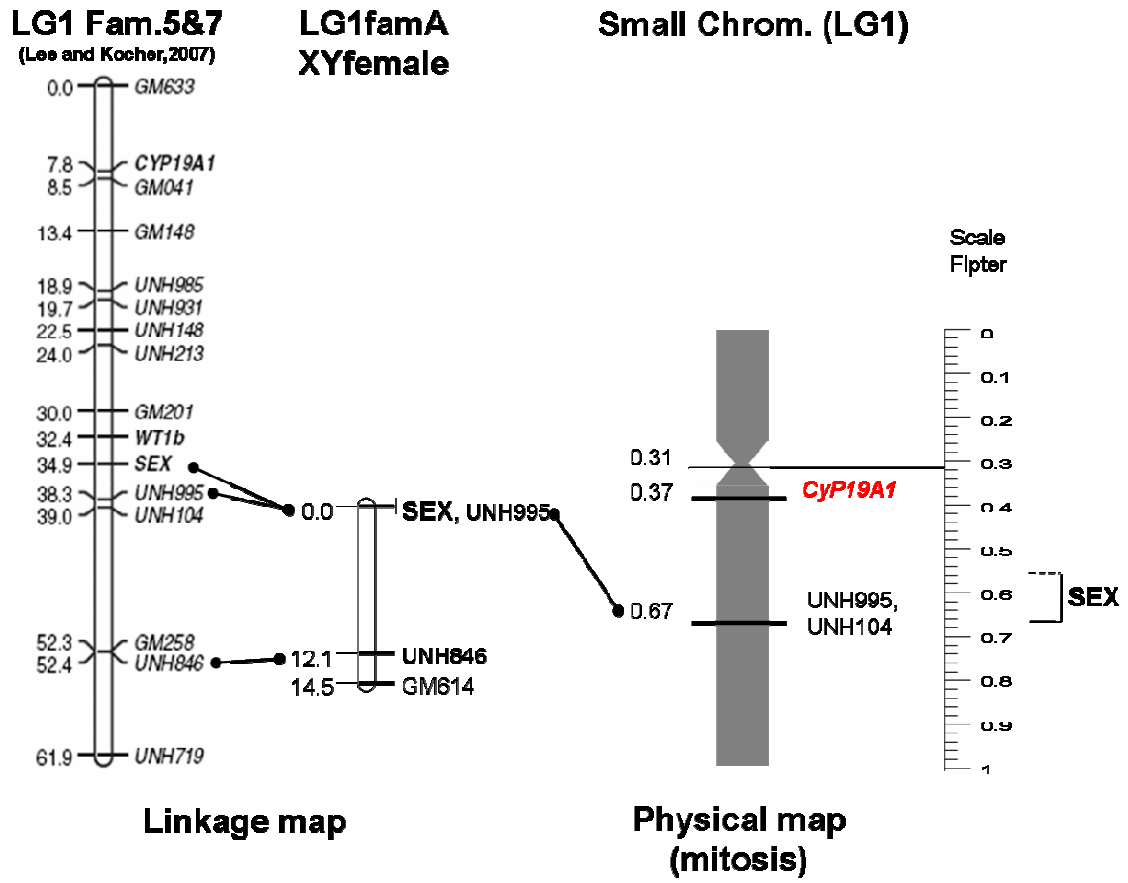

Figure 5.8 A comparison of LG1 and the associated small chromosome in *O. niloticus.* Notice that sex determination locus is proposed to be just immediately after UNH995/UNH104 marker towards the centromere (less than 0.67 FIpter).

The informative markers analysed from LG3 were GM354, UNH168, GM271, GM150, GM128, GM526, UNH982 and *Oni*159. The marker UNH115 did not show informativeness in any of the crosses analysed from Fam.A and B.

Fam.A XY male showed no significant linkage with sex in the two point analysis (Table 5.9). In the case of the XY neofemale, a slight increment in the linkage with sex between the markers GM150 (chi-square= 5.33, P<0.05) and

GM526 (chi-square= 5.89, P<0.05) was observed, with a peak at GM128 (chi-square= 7.36, P<0.01) (Table 5.10).

Despite the statistical significance of linkage with sex from Fam.A XY neofemale with the markers mentioned, their LOD scores were less than 1.6, leading to considering this association as a putative false “nearly positive” and possibly leaving open the possibility of a minor sex associated locus in this region of LG3. Moreover, a sex determining loci on LG3 would not agree with the tight linkage of sex and UNH995 (LG1) observed in the same target fish (Fam.A XY neofemale).

Taking the black blotches into the two point analysis, strong linkage to some of the markers on LG3 for Fam.B XX neomale were found. Examples of grandparents, parents and offspring phenotypes are displayed in Figure 5.9. Black blotches locus was significantly associated with the markers GM128 (chi-square= 23.12, P<0.001) and GM526 (chi-square= 23.12, P<0.001) with LOD scores of 5.50 in both cases (Table 5.11).

Fam.B XX female was compared in a two point analysis with the UNH168 being a marker linked in all the LGs created from LG3. It demonstrated stronger linkage with GM271 (chi-squre= 4.59, P<0.05) than GM150 chi-square= 3.00, n/s) and UNH982 (chi-square= 2.46, n/s) although LOD scores were very low (less than 1.01) in the three cases.

Goodness-of-fit analysis was run for every marker mapped, demonstrating no significant deviation in the segregations and followed Mendelian expectations.

Table 5.9 Fam. A, XY male crossed with clonal line A XX female (D53A x FE7A(c)). Informative markers from LG3 (Lee *et al.,* 2005) and the new marker *Oni*159 showing genotypic proportions of males and females of *O. niloticus*. Significance of linkage between sex and markers was tested by chi-square with 1 degree of freedom, and LOD score considering the maximum likelihood.

Marker	SIRE	DAM	PROGENY			X^2 [1] linkage	LOD score (Z_{max})	N
			Genotypes	Female	Male			
GM354	128/137	128/128	128/137	16	12	0.320	0.069	50
			128/128	15	7			
UNH168	168/170	168/168	168/170	16	11	0.183	0.039	49
			168/168	15	7			
GM271	213/175	213/213	213/175	13	9	0.818	0.178	44
			213/213	16	6			
*Oni*159	167/189	167/167	167/189	15	5	0.555	0.120	45
			167/167	15	10			
GM150	198/206	206/206	198/206	14	12	1.280	0.279	50
			206/206	17	7			
GM526	257/267	257/257	257/267	17	11	0.020	0.004	49
			257/257	13	8			
UNH982	126/128	126/126	126/128	12	7	0.090	0.019	44
			126/126	16	9			

Table 5.10 Fam. A, clonal line A XX neomale crossed with XY neofemale (F3EE x F64C(e)). Informative markers from LG3 (Lee *et al.*, 2005) and the new marker *Oni*159 showing genotypic proportions of males and females of *O. niloticus*. Significance of linkage between sex and markers was tested by chi-square with 1 degree of freedom, and LOD score considering the maximum likelihood.

Marker	SIRE	DAM	PROGENY			X^2 [1] linkage	LOD score (Z_{max})	N
			Genotypes	Female	Male			
GM354	128/128	116/128	128/128	7	9	0.100	0.021	40
			116/128	12	12			
UNH168	168/168	168/170	168/168	11	8	0.900	0.196	40
			168/170	9	12			
*Oni*159	167/167	167/189	167/167	14	14	0.510	0.110	49
			167/189	8	13			
GM150	206/206	198/206	206/206	5	16	5.330 *	1.180	48
			198/206	16	11			
GM128	156/156	156/164	156/156	7	19	7.367 **	1.642	49
			156/164	15	8			
GM526	258/258	258/267	258/258	8	19	5.897 *	1.307	59
			258/267	14	18			
UNH982	125/125	125/127	125/125	14	8	3.930 *	0.866	43
			125/127	7	14			

***P<0.05; **P<0.01.**

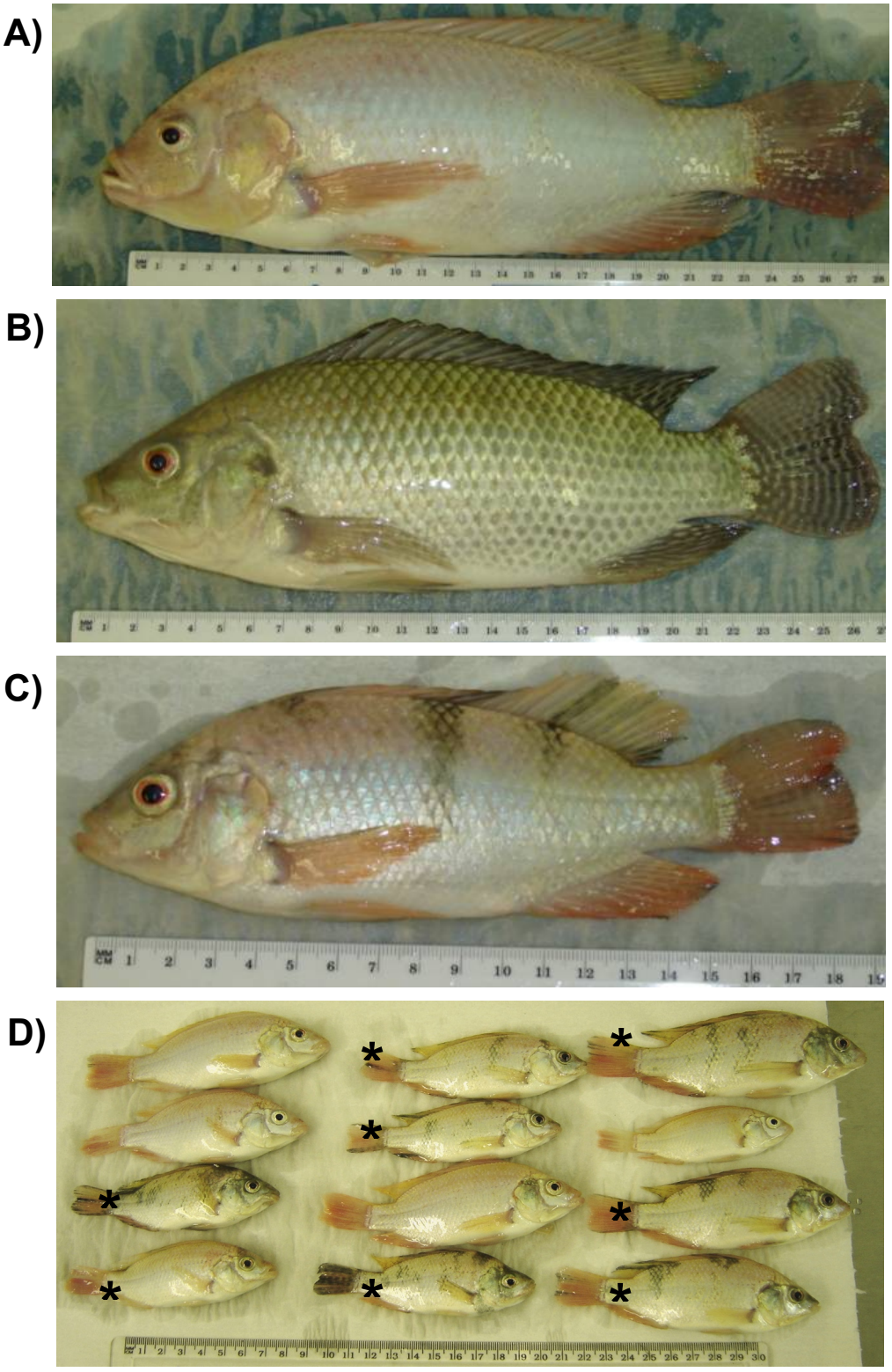

Figure 5.9 Fam. B fish, showing the different skin colourations and black blotches patterns in the three generations. A) An original broodstock (grandparent) from clonal line A XX neomale, red skin colour; B) An original broodstock (grandparent) wild type XX female; C) F1, parent XX neomale with some black stripes on a red skin colour background; and D) F2 offspring XX females, showing complete absence (just red skin colour) and presence of black blotches (fish with asterisk) on different fish. Scales are in centimetres.

Table 5.11 Fam. B, XX neomale crossed with clonal line A XX female (F3EC x 11F3(b)). Informative markers from LG3 (Lee *et al.*, 2005) showing genotypic proportions of males and females of *O. niloticus*. Significance of linkage between skin colour and the markers was tested by chi-square with 1 degree of freedom, and LOD score considering the maximum likelihood.

Marker	SIRE	DAM	PROGENY			X^2 [1] linkage	LOD score (Z_{max})	N
			Genotypes	No Blotch	Blotch			
GM354	116/128	128/128	116/128	12	21	2.083	0.455	48
			128/128	8	7			
UNH168	163/167	167/167	163/167	8	12	0.080	0.017	50
			167/167	12	18			
GM271	175/213	213/213	175/213	6	14	1.975	0.432	41
			213/213	11	10			
OniY 425	213	---	213	15	22	0.320	0.069	50
			---	5	8			
UNH971	292/332	332/332	292/332	15	21	0.020	0.004	49
			332/332	4	9			
GM150	198/206	206/206	198/206	3	20	11.520 ***	2.607	50
			206/206	17	10			
GM128	95/100	100/100	95/100	3	25	23.120 ***	5.504	50
			100/100	17	5			
GM526	240/258	258/258	240/258	3	25	23.120 ***	5.504	50
			258/258	17	5			
UNH982	126/134	126/126	126/134	4	25	20.480 ***	4.815	50
			126/126	16	5			

*****P<0.001.**

Table 5.12 Fam. B, clonal line A XX neomale crossed with XX female (50BF x FCE1(b)). Informative markers from LG3 (Lee *et al.*, 2005) showing genotypic proportions of the marker UNH168 with respect to GM271 and GM150 and UNH982 in *O. niloticus*. Significance of linkage between UNH168 and the other markers was tested by chi-square with 1 degree of freedom, and LOD score considering the maximum likelihood.

Marker	Parents	Genotypes	UNH168 (allele 164)	UNH168 (allele 168)	X^2 [1] linkage	LOD score (Z_{max})	N
UNH168	Sib	168/168	---	---	---	---	---
	Dam	164/168	---	---			
GM271	Sib	213/213	11	17	4.591 *	1.013	49
	Dam	175/213	15	6			
GM150	Sib	206/206	6	11	3.000	0.658	48
	Dam	188/206	19	12			
UNH982	Sib	128/128	9	13	2.469	0.540	49
	Dam	128/166	17	10			

***P<0.05.**

Once the markers were assembled into a single linkage group or broken linkage groups, LG3 from Fam.A XY male and neofemale, and Fam.B XX neomale and female were graphically compared with the F2 hybrid linkage group previously created by Lee *et al.* (2005). Figure 5.10 shows only the markers that were linked with at least one marker from the same LG. Informative markers from LG3 Fam.A XY male were integrated into two groups, one with *Oni*159 and GM354 and the other with UNH163, GM150, GM526 and UNH982. The first group is proposed to be in the order mentioned due to the references from the physical map, and the second group showed similar order but with different distances when compared with the F2 hybrid map such as zero cM (no recombination events) between GM526 and UNH982 in the newly map against 11 cM between the same markers in the F2 hybrid map.

LG3 markers from Fam.A XY neofemale were placed in three groups: *Oni*159 and GM354, UNH168 and GM150, and Gm128, GM526 and UNH982, distributed along the LG3 as listed according to the F2 hybrid map. It is believed that *Oni*159 must be located at the beginning of LG3 due to its physical position along chromosome 1 (previously mapped by Boonphakdee, 2005) at Flpter= 0.85, and because of the low probability of association of GM354 and *Oni*159 with the nearest possible marker (UNH168) in the LG. They failed to assemble into a single linkage group as observed in the map from Fam.A XY male. LG3 markers from Fam.B XX neomale were integrated into a single group (total of 78 cM) of five markers and the black blotches locus (60 cM), localised at the same distance between GM128 (44 cM) and GM526 (76 cM). Finally, LG3 from Fam.B XX female comprised 4 markers assembled in a single LG and with 96 cM in total. GM271 only was mapped in this last family, being both, an inverted

and a longer distance from UNH168 compared to LG3 from the F2 hybrid map. (Figure 5.10)

Maximum possible LOD scores used for the creation of the four LGs described from Fam.A XY male and neofemale and Fam.B XX neomale and female were 2.9, 2.7, 1.8 and 0.65 respectively. The same LOD scores applied for the creation of LG1s in the previous section (5.4.2).

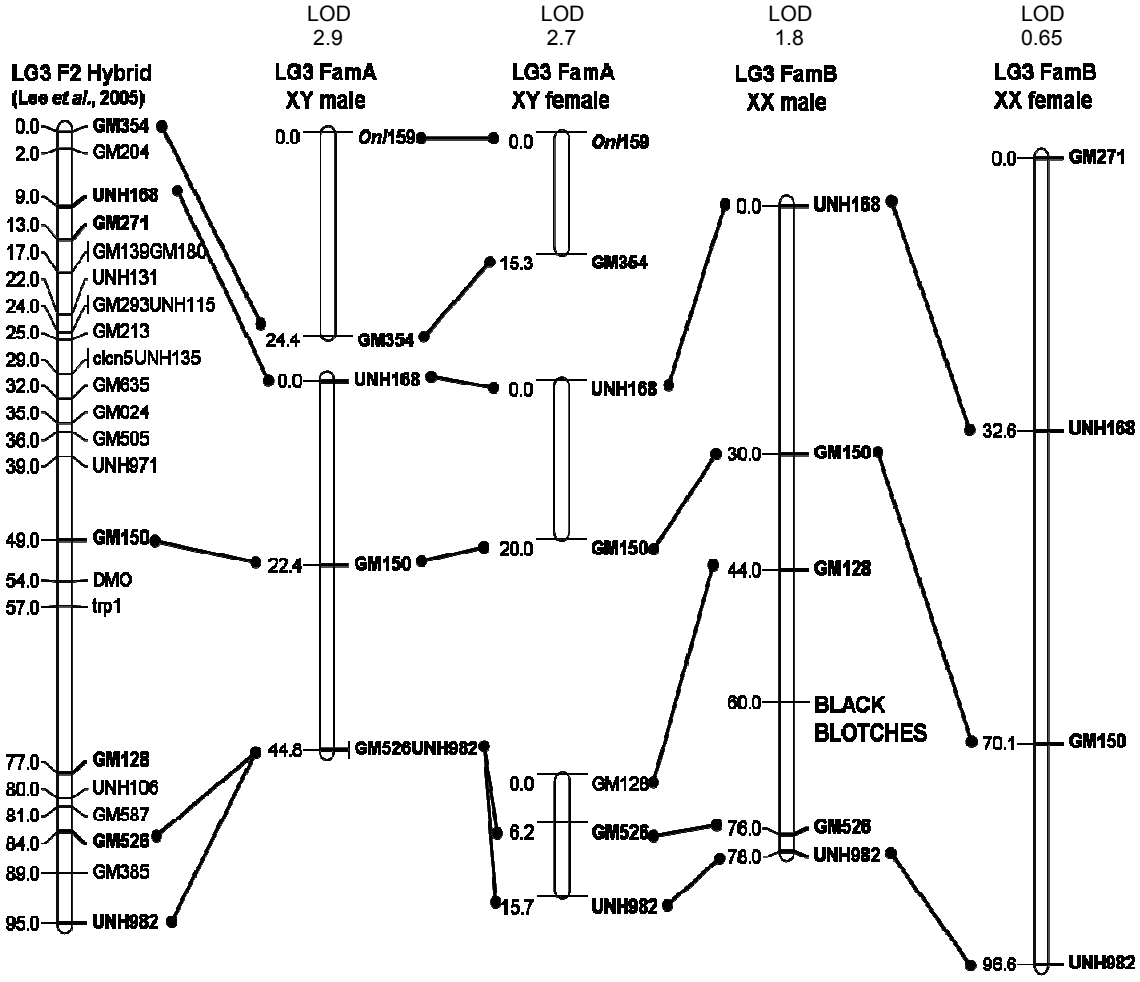

Figure 5.10 Markers from LG3 linked at LOD range 0.65-2.9 for the four different linkage groups newly created, and comparing them with LG3 map developed by Lee *et al.* (2005).

Furthermore, LG3 from Fam.B XX neomale was taken for a comparison with the LG3 physical map associated to chromosome 1 in Nile tilapia (previously described in Chapter 3). This comparison helped to anchor the black blotches locus on the physical map. Considering previous observations from Karayucel *et al.* (2004) who estimated the localisation of the red locus (black blotching on its Rr heterozygous expression) at 4.8 cM distance from the centromere, it was possible to orientate this distance on the large arms of chromosome 1 with the findings from Fam.B XX neomale LG. Taking F2 hybrid map and Fam.B XX neomale, black blotches locus is postulated to be located between the markers GM128 (77 cM or 44 cM respectively) and GM526 (84 cM or 76 cM respectively) with an exact position at 80.2 cM in the F2 hybrid map and 60 cM in the Fam.B XX neomale map (Figure 5.11). Because of the tight physical proximity of the markers located in this part of the LG, black locus is estimated to be located in a physical range of Flpter= 0.06 between Flpter= 0.20 (GM128) and Flpter= 0.14 (centromere) in Nile tilapia with closer proximity to GM128. Finally, the marker UNH982 is proposed to be physically located on the small arm of chromosome 1.

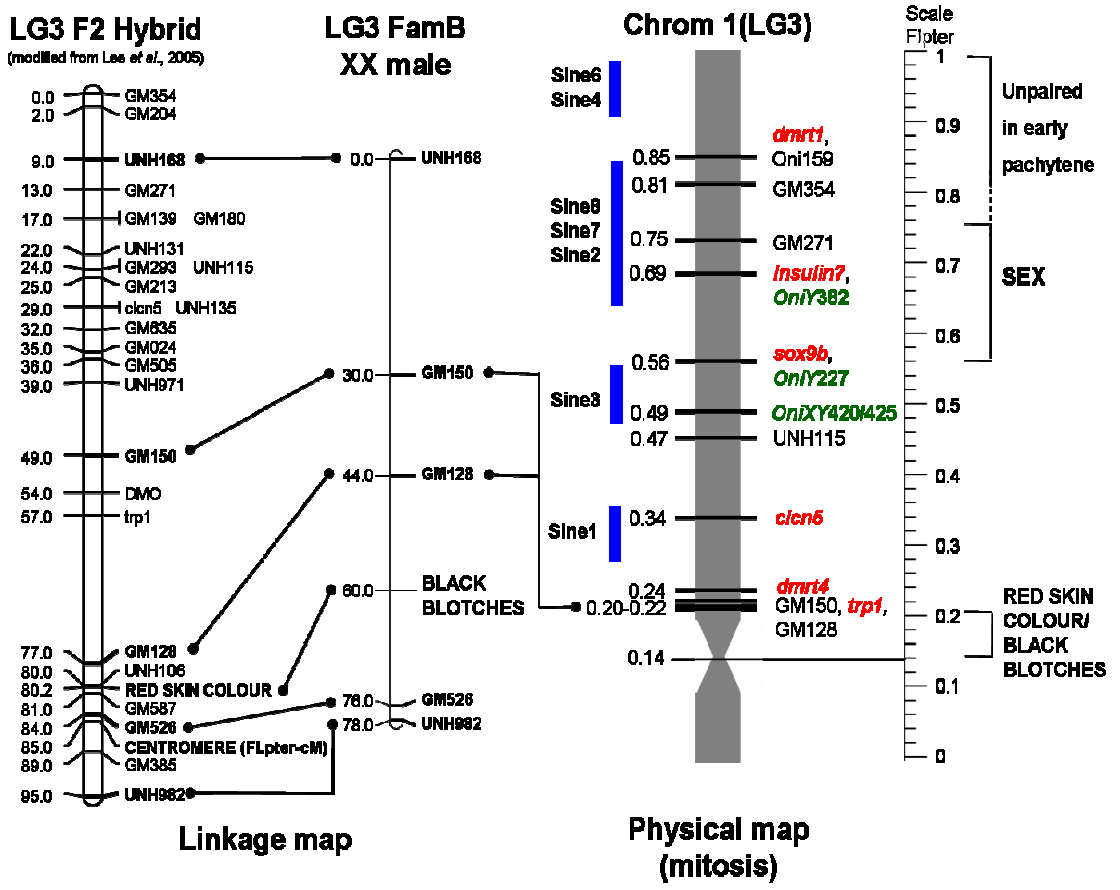

Figure 5.11 A comparison of LG3 and the associated chromosome 1 in *O. niloticus.* Centromere is proposed to be localised at 85 cM after Flpter conversion from meiotic measures (Chapter 3) and the Red/Black blotches locus at 4.8cM apart from the centromere (80.2cM) according to Karayucel *et al.* (2004). This locus position is compared then with an XX neomale linkage map and a physical map described in Chapter 3. Locations of flanking markers to black blotches locus are compared as well. Notice the anchoring of dmrt1 & *Oni*159 (Flpter= 0.85) at the subtelomeric region of the q arm.

5.4.4 Effect of phenotype and genotype on recombination

It was difficult to make a general comparison between males and females and XXs from XYs due to the variation in the successful (informative) markers mapped. Moreover, the genetic distances obtained from each LG have to be considered with care, given the low LOD scores (below 3.0) obtained during the construction of LGs.

General trends were observed such as the slight contraction between UNH995 and UNH846 in the terminal part of the LG1 on Fam.A XY male and neofemale of F2 hybrid map (Lee *et al.,* 2005) but not against the Fam.5&7 map (Lee and Kocher, 2007; Lee *et al.,* 2003). On the other hand, an expansion in the upper part of the LG1 map between GM633 and UNH995 from Fam.B XX female when compared with the F2 hybrid map and the Fam.5&7 map.

In the case of LG3 maps, taking the F2 hybrid map (Lee *et al.,* 2005) as a reference again, Fam.A XY male showed a general contraction between UNH163 and UNH982 (long arm and putative short arm). Fam.A XY neofemale showed a contraction in the first part of the map between UNH168 and GM150 (middle of the long arm), and an expansion between GM526 and UNH982 (putative short arm). Fam.B XX neomale displayed a contraction between UNH168 and GM128 (middle of the long arm, towards centromere), and evident expansion between GM128 and GM526 (long arm, close to centromere) and an expansion again between GM526 and UNH982 (putative short arm). Finally, Fam.B XX female showed a relatively similar linkage distance between UNH168 and GM150 (middle of the long arm) and a possible general contraction between GM150 and UNH982 (long arm and putative short arm, towards centromere).

5.5 Discussion

5.5.1 Proposed main sex determination locus in Nile tilapia

From the two F1 target parents scored for sex (Fam.A XY male and neofemale), only one parent showed evident link with a sex determination locus (Fam.A XY neofemale) into one of the two putative sex linked groups. The marker UNH995 from LG1 predicted with 100% accuracy the phenotypic sex in Fam.A XY neofemale (F64C). LG1 markers from the Fam.A XY male, despite having common parents (FA3F female x 2ED9 male) with the XY neofemale, showed no relation at all with sex. Looking into the genetic differences that these two target fish could have and following the segregation of alleles, it was possible to observe that the combination of four different alleles coming from the grandparents, gave different degrees of sex associations. This finding suggest that these are different alleles at the sex determining locus with specific strength towards males or females, resulting in a progeny with sex ratio depending also upon the strength (with addition or subtraction effect) of the combination of alleles.

Further work on the allelic strength towards sex determination (Institute of Aquaculture MSc project; James M. Walton, 2007), reinforced the above observations and also gave extra information on the individual allele influence. Progeny from one more Fam.A XY male (FFE1) and three from Fam.A XY neofemales (F11D, 393D, 52B6) were genotyped, allowing the confirmation of a sex determining locus on LG1 and a better characterisation of allelic influence of UNH995 locus on sex ratios. Figure 5.12 shows the general allele segregation for UNH995 from Fam.A grandparents and F1 parents.

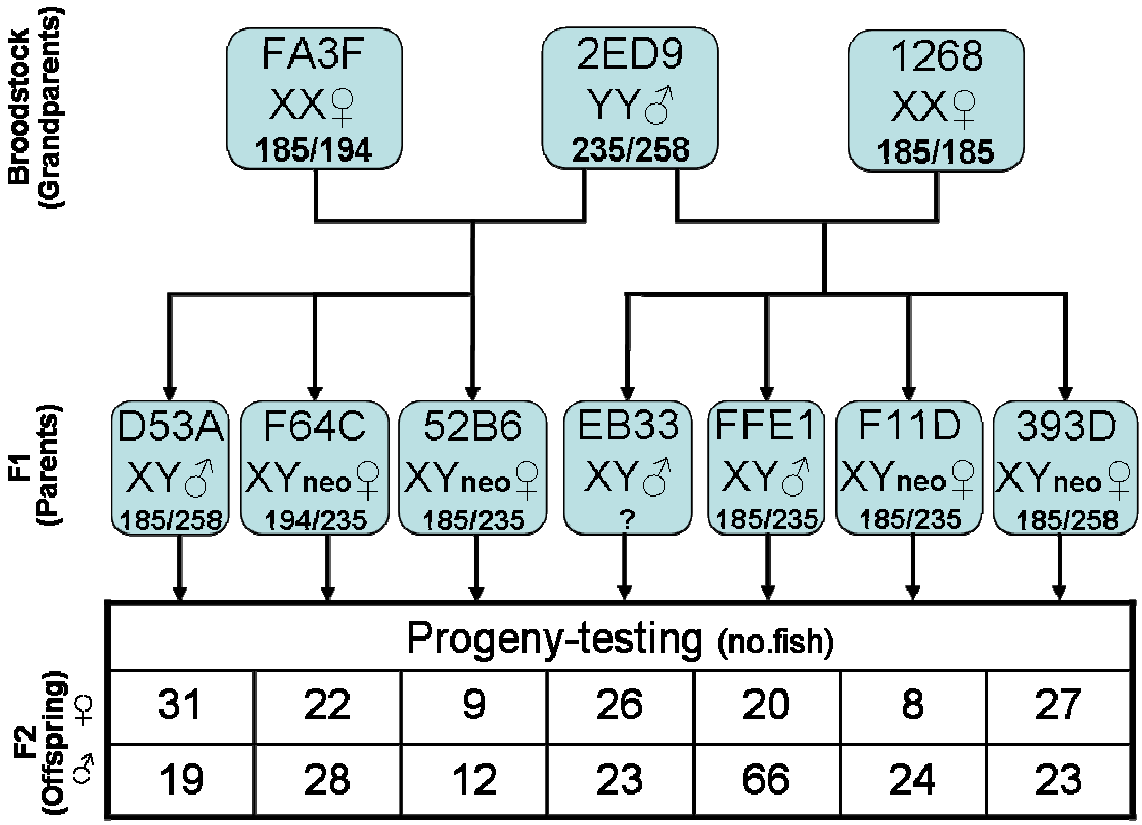

Figure 5.12 Diagram showing UNH995 genotypes (allele composition in bp) for Broodstock and F1 parents (XY males and neofemales) on Family A. F1 parents were subsequently crossed with clonal line no.117 XX male or female with 185/185 allele composition for the generation of F2s. Notice that from EB33, the genotype has not been confirmed (study in collaboration with James M. Walton, 2007).

A clear explanation of this finding is shown in Table 5.13, enlisting the four different allele combinations amplified from UNH995 in the F2 generation. It is possible to distinguish that alleles 194bp and 235bp are strongly associated with female and male determination respectively, and that this association is not significant for the alleles 185bp (in most of the cases) and 258bp. As a result, the sex determining locus appeared to be linked on LG1 only in the four target fish (Fam.A XY target fish F64C, 52B6, FFE1 and F11D) possessing the allele 194bp or 235bp. The other two fish analysed (Fam.A XY target fish D53A and 393D) did not show any significant sex linkage with either LG1 or LG3. A similar

situation was observed by Lee *et al.* (2003), reporting data from three *O. niloticus* families. They found sex-linkage only in two of the three families genotyped, and observed that these two families had several alleles in common, close to the sex determining locus on LG1, but different from the non-sex-related family.

The genotype results from the six target fish were pooled together to provide a better picture of the alleles with respect to sex association (Table 5.14). The pooled data not only enforced the findings mentioned above but also revealed that the different versions (origins) of the allele 185bp have different level of influence towards females (grandparent XX female FA3F versus 1268), reaching the significance level for the allele 185bp coming from FA3F ($P< 0.05$) but not from 1268 (n/s). The strong "allele competition" was evident in comparing two crosses: 1) XX female FA3F × YY male 2ED9 gave 5% female progeny, and 2) XX female 1268 × YY male 2ED9 gave 0% female progeny. With all this information available for family A, it is possible to predict the allele compostion for other F1 members of the family. For example, it is likely that XY male EB33 (see Figure 5.12) has the genotype 185/258 as its F2 progeny segregates for sex in 1:1 ratio.

This level of understanding could lead to a more efficient allelic segregation of beneficial traits (enhancement) such as sex in *O.niloticus* for commercial production. It has been demonstrated in *O. aureus* that it is possible to fix the relevant male sex determining factors involved in Z chromosome and other genetic factors (autosomal alleles) in a neofemale line after five successive generations (Desprez *et al.,* 2003; Lee *et al.,* 2004).

Table 5.13 UNH995 from Fam.A XY males and neofemales showing the different levels of allele associations with sex. F64C, 52B6, FFE1 and FF1D target fish presenting the 235 allele, showed significant linkage with sex determining locus. 185bp allele (from clonal line no.117 XX male or female) is common for all genotypes and the allele coming from the given target fish is displayed in bold.

Family A XY male, UNH995			
D53A Progeny	**Females**	**Males**	**X^2 [1] 1:1**
185/**185**	16 (36.35%)	9 (20.45%)	1.96
185/**258**	12 (25.75%)	7 (20.45%)	1.32
Family A XY neofemale, UNH995			
F64C Progeny	**Females**	**Males**	**X^2 [1] 1:1**
185/**194**	21 (46.66%)	0	21.00 ***
185/**235**	0	24 (53.33%)	24.00 ***
Family A XY neofemale, UNH995			
52B6 Progeny	**Females**	**Males**	**X^2 [1] 1:1**
185/**185**	7 (33.33%)	1 (4.76%)	4.50 *
185/**235**	2 (9.52%)	11 (52.30%)	6.23 *
Family A XY male, UNH995			
FFE1 Progeny	**Females**	**Males**	**X^2 [1] 1:1**
185/**185**	9 (20.93%)	13 (30.23%)	0.73
185/**235**	0	21 (48.83%)	21.00 ***
Family A XY neofemale, UNH995			
F11D Progeny	**Females**	**Males**	**X^2 [1] 1:1**
185/**185**	6 (19.35%)	7 (22.58%)	0.08
185/**235**	1 (3.22%)	17 (54.83%)	14.22 ***
Family A XY neofemale, UNH995			
393D Progeny	**Females**	**Males**	**X^2 [1] 1:1**
185/**185**	15 (31.91%)	11 (23.40%)	0.62
185/**258**	12 (25.53%)	9 (19.14%)	0.43

***P< 0.05, ***P< 0.001**

Table 5.14 Pooled data from UNH995 allele composition, considering the 6 families with allelic information shown in Figure 5.12. 185bp allele (from clonal line no.117 XX neomale or female) is common for all genotypes and the allele coming from the given target fish is displayed in bold.

GENOTYPES	Females	Males	X^2 [1] 1:1
185/**235**	3	73	64.47 ***
185/**258**	24	16	1.60
185/**194**	21	0	21.00 ***
185/**185** FA3F version	23	10	5.12 *
185/**185** 1268 version	30	31	0.02

***P< 0.05, ***P< 0.001**

As mentioned in the results section, Fam.D XY males were not genotyped. However comparing their sex ratios with Fam.A XY males or neofemales, it is possible to observe skewed sex ratios with significant deviations from 1:1 in some crosses (eg. Fam.A XY males: 1435 x FFE1 with P< 0.001 or Fam.A XY neofemales F64C x F3EE with P< 0.05), and even among brothers having the same phenotype. On the contrary, all crosses from “control” Fam.D XY males (that came from a normal cross XY male x XX female) showed no significant differences from 1:1 sex ratios. This observation suggests that the major male-determining locus has also different levels of penetrance on each Y chromosome depending on family background. However, different levels of autosomal influences on different families can not be discounted.

The hypothesis of a major sex determining gene involved in the monofactorial sex chromosome determination system of tilapia as previously proposed is widely supported from previous works (Ezaz *et al.,* 2004c; Lee *et al.,* 2003; Mair *et al.,* 1997; Shirak *et al.,* 2006) even when there is still not a consensus about the location of this gene. Both XY and WZ system have been demonstrated not to be allelic (Lee *et al.,* 2004) despite their strong association with sex. From this study it is suggested to consider the effect of different sex determination thresholds operating in tilapia, associated with the strength and interaction of specific alleles or genes as described in Table 5.15. This effect on sex could also be called a threshold dichotomy (Mittwoch, 2006), defining males or females as a result of the interaction of multiple genes with different levels of quantitative effects that express depending on their genetic and environmental background as observed in dragon lizard (Quinn *et al.,* 2007) and also in tilapia (Abucay *et al.,* 1999; Baroiller and D'cotta, 2001; Mair *et al.,* 1991a). Nevertheless, a lead sex determining gene takes the critical role, towards male (XY system) or female (WZ system) as no intersex tilapia has been observed without the use of hormones in any of the progeny analysed in this and previous studies (e.g. Mair *et al.,* 1994; Melard *et al.,* 1994).

In an F2 hybrid generation from XY male *O. niloticus* x WZ female *O. aureus* an association of sex determination locus with several markers from LG3 was reported (Lee *et al.,* 2005). The genotypic sex of the F2 hybrids were expected to be XX?-WZ female, XY?-WZ female, XX-ZZ male and XY-ZZ male, demonstrating more influence of WZ system (associated to LG3) on phenotypic sex, although Lee *et al.* (2005) observed and analysed more females than males from the F2 progeny (1.51:1 female:male).

Since an earlier autosomal theory (Avtalion and Hammerman, 1978) does not explain all sex ratios observed in tilapia hybrids (Desprez *et al.*, 2006). Ezaz *et al.* (2006) suggested that the tilapia sex determination model gives an example of a transition system (or recently evolved system) between sex chromosomes, where sex specific sequences in one species are autosomal in sister species. Another well studied species of a clear interaction between XY-WZ systems is illustrated with the platyfish (*Xiphophorus maculatus*), demonstrating X, Y, W and Z chromosomes within the same population (Volff and Schartl, 2001). Different combinations of these sex chromosomes gave different phenotypic sexes, proposing that sex is determined by a dosage of sex determining and regulatory genes located in different sex chromosomes and autosomes (Volff and Schartl, 2001).

From the specific crosses observed from Fam.A XY male and neofemale, it is possible to say that the allele 235bp at UNH995 is a marker for the Y chromosome and the 194bp is a marker for the X chromosome. Table 5.15 proposes a preliminary model for the dosage sex determining effect, combining XY-WZ systems according to other previous observations (e.g. Cnaani *et al.*, 2007; Lee *et al.,* 2003, 2004; Mair *et al.,* 1991b; Melard *et al.,* 1994), where Y chromosome plays a stronger dominant male determiner over Z, and W chromosome is a stronger female determiner (or male repressor) over X. Alternatively, if a YY or XX genotype with different alleles is present, one allele still could show stronger dominance than the other (such as YY 2ED9, 235/258 alleles); but having the same allele, no significant differences should be observed (such as XX 1268, 185/185 alleles).

A final LG1 and LG3 map comparison between males and females from *O. aureus* and the F2 hybrid with *O. niloticus* (Lee *et al.,* 2004), and the new maps presented here and in Lee *et al.* (2003) in *O. niloticus*, suggests a dominant WZ system (LG3) over the XY system (LG1) as suggested before by Lee *et al.* (2005) where the putative Y chromosome (dominant male determiner) did not show a stronger association with sex in the presence of the putative W chromosome (dominant male repressor) on a single hybrid cross, as observed in the pure *O. aureus* linkage maps (Lee *et al.,* 2004), although an epistatic interaction was observed on LG3.

Table 5.15 Genetic dosage effect from different chromosome/allele combination in tilapia sex determination from *O.aureus*, *O. niloticus* and hybrids. Scale 1 to 7 goes from the strongest to the weakest effect towards male. Genotypes in BOLD represents the chromosome(s) (or allele(s)) with the apparent highest sex determination influence.

Dosage Effect Scale	Sex Determination Genotype (UNH995 alleles)	% of males	Comments	Reference
1	X**Y**-ZZ Hybrid	100%	**Y** allele, 189bp from UNH104, LG1.	Lee *et al.*, 2004, 2005.
2	**Y**Y(**235bp**/258bp)	95-100%	E.g.Broodstock Fam.A 2ED9.	Present study; Mair *et al.*, 1997
3	XX-**ZZ**-**autosome(s)** Hybrid	52-100%	Depends upon the purity of stocks. With **ZZ?-autosome(s)** (60-100% males)	Mair *et al.*, 1991. Melard 1994. Hulata *et al.*, 1995.
4	X**Y**(194bp/**235bp**)	60-80%	E.g.F1 Fam.A.F64C	Present study
5	**XY**(**185bp/258bp**)	42-57%	E.g.Fam.A.D53A. Also **W**Z (187bp/193bp) UNH131, LG3	Present study; Lee *et al.*, 2004
6	XY-**W**Z Hybrid	0% (all females)	**W** allele, 187bp from UNH131, LG3.	Lee *et al.*, 2004, 2005.
7	**XX**(**185bp/185bp**) or XX-**W**Z Hybrid	0% (all females)	E.g. All crosses from Fam.B. Also **W** allele, 187bp from UNH131, LG3. **WW?** (187bp/187bp)	Present study; Lee *et al.*, 2004, 2005.

5.5.2 Proposed black blotches locus in Nile tilapia

In the case of the recessive black blotching (or dominant red skin colour) locus, the allele association was easier to identify as informative markers flanked (on both sides) the locus under study. Fam.B XX neomale linkage analysis was demonstrated to have a strong association to the black blotching gene, located in the middle (60 cM) between the markers GM128 (44 cM) and GM526 (76 cM) on LG3, whilst Lee *et al.* (2005) suggested a location on the same group but between Trp1 gene (57 cM) and GM128 (77 cM) in the F2 hybrid map. Nevertheless, the gene-centromere cM distance calculated by Karayucel *et al.* (2004) agree with the present findings, having a slightly closer proximity to GM128 (see section 5.5.5).

During black blotches scoring, only absence or presence of this characteristic was considered, iirrespective of the grade of blotching presented. McAndrew *et al.* (1988) noticed that the heterozygous fish presented variable black blotch patterns from 0% (undetectable) to 24.6% of the fishes' surface including fins making the scoring inaccurate. However, it was observed that the family score in this research showed most of the time clear differences between blotching and no blotching making easy the scoring process and even when the blotching was minimal in some fish, it was counted in the black blotching pool. This suggests that the R allele had a relatively low dominance considering that the degree of blotching increases as the level of dominance from the R allele decreases in the cross (McAndrew, 1988).

By comparing the allelic segregation of two loci carried on the same chromosome (e.g. A and B), it is possible to predict how close these loci are to each other based on the frequency that the maternal A,b) and paternal (a,B)

copies of the two alleles are exchanged by meiotic recombination to produce novel genotypes (A,B or a,b). If the two loci are very close to each other or tightly linked, the recombination will be infrequent. On the other side, if the two loci are distant each other, the chances of recombination will be higher and having both genes segregating independently (Miesfield, 1999). In the particular case of GM128 (95bp/100bp) and GM526 (240bp/258bp), the non-recombinant allele combination 95bp/240bp showed association in 73% of the cases reported with blotching and the non-recombinant 100bp/258bp showed no association with blotching in 70% of the cases. The other two novel genotypes produced (recombinant 95bp/258bp and 100bp/240bp) accounted for the minority of the cases with an even association between blotching and no blotching (see Table 5.16).

Thus, it is demonstrated then that a red dominant colour allele (R) and a red recessive colour allele (r) are playing a major role in red skin colour and black blotching. A dominant red (RR) results in an absence of black blotches (in most of the cases) and expression of the wild type is present with a complete grey skin colour (rr) or black blotches (Rr, in most of the cases). These observations are supported by previous studies (Hussain *et al.,* 1994; McAndrew *et al.,* 1988) suggesting that red skin colour is a dominant characteristic but with variable penetrance or expressivity, presenting different black blotching coverage.

Despite the red skin colour/black blotches locus has been linked into the LG3 (associated to one of the putative sex linked chromosomes, chromosome 1) (present study and Lee *et al.*, 2005), previous genetic analysis showed that

red skin colour is an autosomal dominant in *O. niloticus* (McAndrew *et al.,* 1988). Moreover, in several pooled meiotic gynogenetic families it has been demonstrated that there is a close genetic linkage between the red locus and one of the two autosomal genes causing female-to-male sex reversal but with fairly low penetrance, and a weak linkage with the main sex determination locus (Karayucel *et al.,* 2004). These findings, together with the statements discussed in the previous section about sex determination detected on LG1 make clearer that neither chromosome 1 nor the red locus play the main role in sex determination in Nile tilapia, although these may have a secondary (modifying) role.

Expanding further the observations of Karayucel *et al.* (2004) and Ezaz (Ezaz *et al.,* 2004c) in relation to the autosomal sex reversal, it was suggested that the two putative autosomal genes could cause female-to-male and male-to-female reversion and that there is a significant (positive) correlation with temperature sex determination (TSD) (Abucay *et al.,* 1999; Baroiller *et al.,* 1996; Kwon *et al.,* 2002). However, considering Cnaani *et al.* (2004) and Shirak *et al.* (2006) observations on sex determination, sex specific mortality and low temperature tolerance on LG23 detected in *Oreochromis* hybrids (*O. aureus x O. niloticus* and *O. aureus x O. mossambicus* respectively), it is not possible to suggest yet that chromosome 1 may be one of the autosomal chromosomes involved in TSD until further linkage comparison studies on LG23 using pure *O. niloticus*.

Table 5.16 GM128 and GM526 from Fam.B XX neomale, showing the different allele associations with absence and presence of black blotches.

Family B XX neomale, GM128 & GM526		
11F3 Progeny	**Blotch**	**No Blotch**
95/240	22 (73%)	0
100/258	2	14 (70%)
95/258	3	3
100/240	3	3
Total	30	20

5.5.3 Comparisons of early development on fish families

Progeny from original broodstock for Fam.A, B and D, generally showed higher degrees of fecundity, fertilisation, fry survival and lower abnormality than broodstock Fam.C. This profile was similarly described for progeny from F1 generation in the four families with some exceptions.

Progeny from F1 Fam.A had the highest percentage of abnormal fry, followed by Fam.D, Fam.B and Fam.C in decreasing order. The highest percentage of fertility and survival were observed on Fam.D, followed by Fam.B, A and C subsequently. However, the differences were not significant (p=0.05) at a given threshold. Moreover, the percentage of survivals at the different stages were comparable with the figures obtained in diploid control batches from *O. niloticus* XY neofemales from the University of Stirling strains in previous crosses (Ezaz *et al.,* 2004a).

The only significant differences were observed between males and females ($p < 0.05$) within the families, with the male test crosses having higher numbers in fecundity, fertility and survival, particularly associated to the better performance from the clonal line XX females used to generate F2s, with bigger body size, larger number of eggs/spawn and slightly bigger eggs than females test crosses. Moreover, the reduction in early survival rate in Fam.C on progeny from both YY males and neofemales relative to the other three families (despite began with similar number of eggs spawned) probably indicate a genetic depression in early development viability from crosses involving at least two Y chromosomes in the combined parental genotype, e.g. XY × YY, XX × YY or XY × XY.

Nevertheless, as Mair *et al.* (1997) and Beardmore *et al.* (2001) proved that YY males are as viable and fertile as XY males, it is possible that a particular genetic effect (e.g. inbreeding) is reflected in the reproductive fitness of Fam.C.

These general observations in early development indices apparently had no implications in the sex ratios observed, except that the lower number of progeny tested per cross from Fam.C, inevitably hampers the reliable identification of YY F1 parents.

5.5.4 Characterisation of linkage group 1 and 3

Few but relatively well distributed markers along LG1 and 3 were selected and linked, the main purpose being to illustrate differences in recombination between the markers when comparing male and females with the same

genotype and different phenotype or vice versa. Unfortunately, due to problems (only one or none informative markers to map and/or skewed progeny sex ratios) with family C and D, only Fam. A and B were taken further, considering anyway that target animals with the representative genotypic and phenotypic combinations, male and females with XY or XX genotype, were still available for a deep analysis.

From the pool of LGs 1 and LGs 3 from Fam A and B, and with the resolution given by the markers used, it is possible to suggest that there is some kind of stronger influence in the total lengths from the genotype rather than the phenotype, being shorter on Fam.A XY cross than Fam.B XX cross; and observing only phenotypes, female maps tend to be shorter than males as observed between LG1 Fam.A male and neofemale (19.3 cM and 14.5 cM respectively), and between LG3 Fam.B neomale and female (75.0 cM and 64.0 cM respectively) from UNH168 to UNH982. This last observation seemed to be reinforced by the linkage maps generated by Walton (2007), with the LG1 mapping of three markers close to the sex determining locus from four other families from Fam.A (total lengths: 52B6, 49.1 cM; FFE1, 101.1 cM; F11D, 37 cM; 393D, 64.4 cM; see Figure 5.12 for family reference), having a significant difference (chi-square1:1= 17.22, P< 0.001) between males and females, with larger maps from phenotypic males. Nevertheless, these observations are still not conclusive due to the relatively incomplete maps developed.

Stronger influence from phenotype rather than genotype on SC total length was found in crested newts (Wallace *et al.,* 1997), mice (Lynn *et al.,* 2005) and tilapia (Campos-Ramos, 2002). This last author observed longer

SCs in females than males or neomales in three species of tilapia: *O. niloticus, O. mossambicus* and *O. aureus*. Considering that SCs (pachytene stage of meiotic chromosomes) are a better linear representation for cM distances than mitotic chromosomes, as discussed in Chapter 3, the present results show the opposite behaviour with respect to the phenotype, having shorter linkage maps in females and longer in males. The explanation could lie on the slight contraction in the flanking region mapped with the sex determination locus (LG1 Fam.A XY neofemale) between UNH995 and GM614 and an evident expansion of the marker distances surrounding the black blotches locus (LG3 Fam.B XX neomale) between GM128 and GM526, supporting the idea of a genetic influence on these specific regions rather than a phenotypic influence. The effect of contraction and expansion on the surroundings of these two loci simply could reflect the normal pericentromeric/ peritelomeric differences between sexes of gonochoristic species (Danzmann and Gharbi, 2001; Lorch, 2005).

The contraction on LG1 Fam.A XY neofemale and expansion of LG3 Fam.B XX neomale are also evident when comparing the LG1 and 3 from Fam.5&7 map (Lee and Kocher, 2007; Lee *et al.,* 2005). Further comparisons with *O. aureus* and *O. niloticus* maps on the black blotching locus on LG3 were not possible since the markers close to the centromere concerning this locus are not anchored in older versions (Kocher *et al.,* 1998; Lee *et al.,* 2004; McConnell *et al.,* 2000).

Suppression in recombination in or around the sex determination locus on LG1 has been demonstrated in other maps when comparing XY male (Lee *et al.,* 2003) with XX females in *O. niloticus* or with the F2 hybrid map (*O. aureus* x

O. niloticus) (Lee *et al.,* 2005). Comparisons with *O. aureus* male on LG1, show also a contraction on flanking markers to the putative sex determination (between GM201 and UNH846) when comparing with female LG1 map, but the overall map size is longer in males than females (2.3%) as it has been demonstrated in the F2 hybrid map (Lee, 2004).

It has been observed in many species that the meiotic recombination rates varies between the two sexes in the sex-specific maps, usually having suppression in recombination in the heterogametic sex (Nei, 1969) such as LGs associated with XY or ZW systems, although when comparing total map ratios from diploid chiasmata species variations do not seem to hold a rule (Campos-Ramos *et al.,* In review; Lorch, 2005). Mammals show sexual differences in sex-specific maps in most cases, such as human (Dib *et al.,* 1996) dog (Mellersh *et al.,* 1997), pig (Marklund *et al.,* 1996) and horse (Swinbume *et al.,* 2006). There are several cases in fish also showing sexual differences in recombination rates such as medaka (Yamamoto, 1964; Naruse *et al.,* 2000), platyfish (Kallman, 1965), rainbow trout (Sakamoto *et al.,* 2000) and other salmonid species (Woram *et al.,* 2003), leading to the segregation of genes in the population with a high hierarchy in the sex determination process (Purdom, 1993).

In the case that sex would be determined by two or more genes, it is necessary in a dioecious organism that these genes are inherited together, avoiding their reshuffle by recombination; otherwise, suppressing the recombination at specific locus (Nei, 1969).

According to Dr. Avner Cnaani (unpublished observations), in tilapia species with female heterogametic sex determination on LG3 such as *O. aureus*, the female map is much shorter than male map. He hardly observed any recombination in female maps between UNH131 and GM354 (Figure 5.13). In the male map, the distribution was similar to the F2 hybrid map presented by Lee *et al.* (2005) but different from the *O. aureus* map presented in Lee *et al.* (2004), e.g. shorter distance between GM354 and UNH115 in males and larger in females despite the *O. aureus* families had the same origin. These evident differences in maps are believed to be artefactual, depending on whether the male and female data were analysed separately on CRIMAP or combined (Dr. Avner Cnaani, personal communication).

These implications lead to consider certain flexibility in the way the data are analysed and the final maps interpreted under the different software programmes.

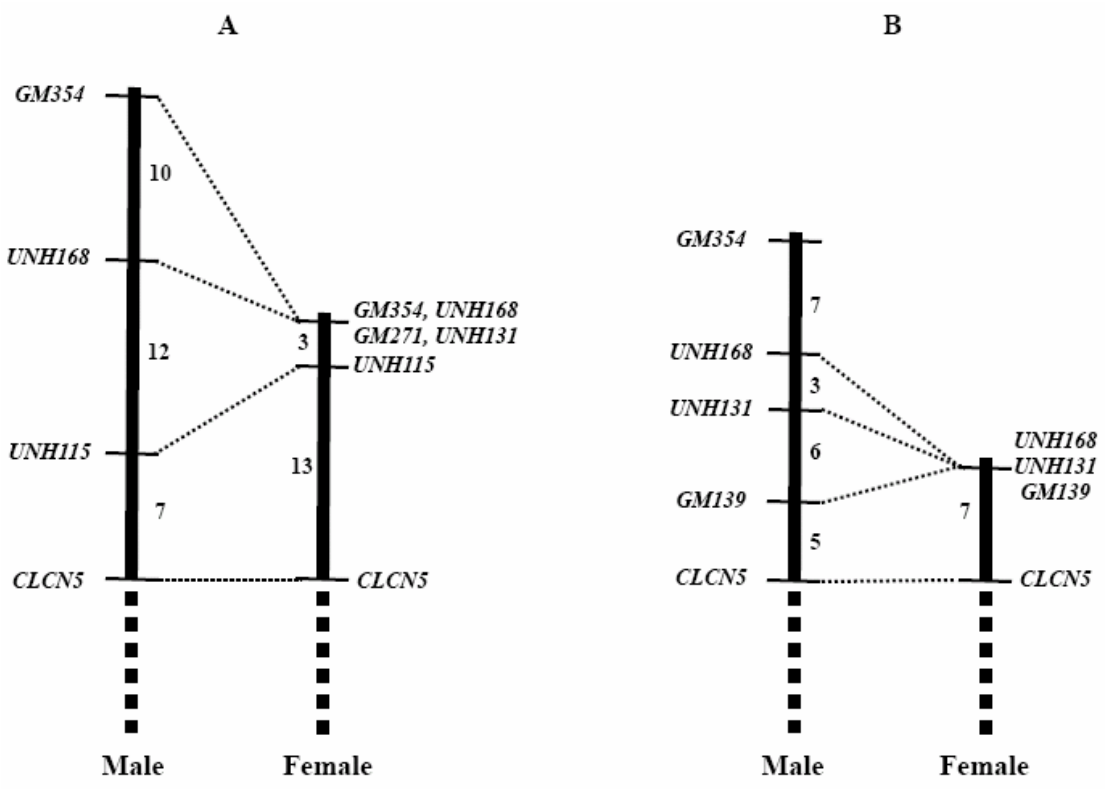

Figure 5.13 LG3 maps comparison developed by Dr. Avner Cnaani (unpublished observations) between males (ZZ) and females (WZ) from two *O. aureus* families (A and B).

During map comparisons, it was observed that certain markers appear in different places such as the marker UNH719 (LG1), jumping from the end of the LG (61.9cM) (Lee and Kocher, 2007) to 47 cM on different F2 hybrid crosses (Lee *et al.,* 2005) or the marker GM139 (LG3), from being close to UNH131 at 17cM in the F2 hybrid map (Lee *et al.,* 2005) to 0 cM close to GM354 in an F2 hybrid map (Dr. Bo-Young Lee, unpublished observations) and *O. aureus* map (Lee *et al.,* 2004). Such observations could be explained in *O. tanganicae* linkage map, where two different loci on the same LG were amplified by genotyping of the marker GM139 (Dr. Avner Cnaani, personal communication), suggesting duplication of the locus close to the telomeres on the long arm of chromosome 1. Other research found in particular the microsatellite UNH111

(LG7) to be duplicated in the genome by the amplification of three different alleles in *O. aureus*, *O. niloticus* and their F2 hybrid (Palti *et al.,* 2001).

In the case of UNH719 (LG1) mapping on different positions is explained with the observations made from Fam.A male and neofemale and Fam.5&7 map (Lee and Kocher, 2007). The F2 hybrid map (*O. aureus* x *O. niloticus*) (Lee *et al.,* 2005) demonstrates a chromosomal inversion of the telomeric section of the long arm between UNH719 and GM614, a phenomenon that was also previously observed on LG9 when comparing *O. niloticus* and *O. aureus* (Kocher *et al.,* 1998; McConnell *et al.,* 2000) against the F2 Hybrid map (Lee *et al.,* 2005; Dr. Bo-Young Lee, unpublished observations).

Low LOD (likelihood of odds) scores obtained from the different LGs (Fam.A XY male and neofemale, Fam.B XX neomale and female with 2.9, 2.7, 1.8 and 0.65 respectively) in general reflected longer distances between markers and in two of the cases (LG3 Fam.A XY male and neofemale), markers failed to be linked into a single group as expected. Although, considering previous LG1 and 3 versions (Lee and Kocher, 2007; Lee *et al.,* 2005), it was possible to arrange the markers in an expected order and make general observations. There were also some unexpected orders in the markers linked together. LG1 XX female (LOD 0.65) had a change in the common order between UNH846 and UNH995 with a subsequent reduction in distance between them; also on LG3 XX female (LOD 0.65) the marker GM271 jumped out of the expected range (32 cM from UNH168) and in different order (before UNH168). The Theta function (only one crossover per chromosome arm) was used throughout to enable comparison with previous map versions.

It is important to remark that the final linkage maps arrangements were calculated according to the most probable association after multicomparison, and that if this probability dropped enough between two “adjacent” markers (due to a large recombination events and/or to a genotyping errors), the potential linkage breaks, appearing as independent LGs. Moreover, the overall low LOD scores could be explained by the large genetic distances between the markers (some are more than 20 cM apart), but considering that previous LGs are known (LG1 and LG3), reducing the LOD threshold for the calculation of map distances, most of the corresponding markers were associated with the right group.

A particular behaviour on the LOD score distributions was observed on the LG3 Fam.A XY neofemale, giving a significant difference ($P< 0.01$) in allele distribution of GM128 with respect to sex and a peak LOD of 1.64 whilst LG1 in the same cross clearly demonstrated to segregate alleles from UNH995 with a complete association to sex ($P< 0.001$). This could be an example of a false “nearly positive” LOD score on LG3, confirmed by the presence of a major sex determining gene in LG1, and reflecting the importance of considering a significant LOD threshold for a given gene or QTL. The LOD threshold most widely used for map construction in humans, fish and other animals is 3.0 (e.g. Danzmann and Gharbi, 2001; Dib *et al.,* 1996; Gharbi *et al.,* 2006; Klukowska *et al.,* 2004) and also accepted as a strong association with a QTL; this LOD reflects a $P< 0.001$, expecting a similar normal distribution between LOD and chi-square outputs (Lynch and Walsh, 1998). A comparison of the distribution from both statistical tools was reflected with the sex association on LG1 Fam.A XY neofemale, where the chi-square and LOD scores proportionally increased

as they move closer to the marker UNH995. Lynch and Walsh (1998) also remark that with the use of selected relatives, the power of test for major genes are greatly improved as was observed for sex determination and black blotches locus, compensating the low LOD scores obtained for linkage map construction.

As mentioned above, map distances were calculated using the Theta function, assumes higher interference than kosambi function affected by the chromosome structure and size (LG1 from small submetacentric and LG3 from the subtelocentric chromosome 1 in tilapia), as observed in comparative analysis in salmonids (Danzmann *et al.,* 2005). Tilapia chromosomes are relatively small compared with mammals for instance (Griffin *et al.,* 2002; Neff and Gross, 2001), decreasing the chances of double crossover. Acrocentric chromosomes also have less recombination than metacentric chromosomes. Several studies have shown that in many fish species there is a high level of chiasma interference, presenting one chiasma event per meiosis (Allendorf *et al.,* 1986). This was confirmed in tilapia by Campos-Ramos (2002) who observed no more than one chiasma per bivalent on SCs and especially on chromosome 1 supported by the presence of heterochromatin blocks (causing suppression of recombinations) (Oliveira and Wright, 1998). In this case one simple recombination is considered to suppress any other recombination at the same time on the same bivalent, making a single recombination equal to a single crossover.

5.5.5 Integration of physical and genetic maps from LG1, LG3 and comments on other LGs

Detailed comparisons between newly characterised physical maps and previous versions of physical and genetic maps (e.g. Harvey *et al.,* 2003b; Lee *et al.,* 2005; Martins *et al.,* 2004) have been described in Chapter 3. Taking into consideration the previous association made for LG1 with a submetacentric small chromosome and LG3 with subtelocentric chromosome 1 in Nile tilapia, in the present chapter, the new LG1 and LG3 developed with linkage to sex determination and black blotches loci respectively were anchored to the existing physical map.

According to the LG1 Fam.A XY neofemale, the main sex determination locus in *O. niloticus* is proposed to be at FIpter=0.67 on the physical map close to the marker UNH995, but according to the F5&7 map (Lee and Kocher, 2007) the sex gene should be at FIpter= 0.61 (as deducted from 34.9 cM position recorded for this marker). Then, it could be proposed that the sex locus would be situated around FIpter= 0.67, considering that cM distances are relatively conserved in the middle of chromosomes (Danzmann and Gharbi, 2001; Nachman, 2002) at least between the flanking markers GM201 and UNH846, as observed when comparing males and females (Fam.A XY male and neofemale) (Lee *et al.,* 2003). Nevertheless, a physical location (by FISH) of close by markers on the other side of the proposed sex locus such as GM201 and WT1b gene would be required to get a more accurate estimation.

LG3 Fam.B XX neomale mapped two markers (GM150 and GM128) that were possible to compare on the physical map on chromosome 1. Considering previous FIpter to cM conversion from meiotic observations on the larger

bivalent (Chapter 3), the centromere on LG3 F2 hybrid (Lee *et al.,* 2005) would be situated at 85 cM. Moreover, considering that gene-centromere estimates agree well with a linear map order generated from recombination intervals (Danzmann and Gharbi, 2001), Karayucel *et al.* (2004) postulated a red locus in Nile tilapia at 4.8 cM from the centromere. This locus was then proposed to be at 80.2 cM, agreeing with Fam.B XX neomale map. On the other hand, if the centromere is anchored on the newly map at 4.8 cM far down from the located black blotches locus, it would be located at 64.8 cM on this map, then at least the marker UNH982 could be considered to belong to the small arm of the chromosome 1 and possibly the marker GM526 if the new distances are accurate (see also the proximity of GM529 and UNH982 on LG3 Fam.A XY male and neofemale.

The black blotch locus is clearly on or very close to a hot spot cluster of recombination (described by Nachman *et al.* (2002) as cluster of small hot spots of approximately 1 Kb length each with recombination rates noticeable higher than the overall genome rate). Observing a clear map expansion in the different LG3 genetic map version but a contraction on the physical map (GM150 and GM128 and other genes tightly close each other). It confirms as well that near the centromere on chromosome 1, the crossover rate tends to be higher.

Despite the fragmentation of Fam.A XY male and neofemale linkage maps, *Oni*159 marker could be possibly anchored between 15.3 and 24.4 cM in the beginning of the hybrid map (before GM354), a range where it would be expected (identified on a BAC clone Flpter=0.85 containing dmrt1 gene,

according to Boonphakdee, 2005). The linkage between GM354 and *Oni*159 was observed on the two crosses from Fam.A genotyped with these two markers.

Finally, it is relevant to mention a different approach taken for Nile Tilapia LGs orientation with respect to their centromere. Centromere positions from each of the LG have been estimated graphically by Dr. Thomas Kocher (unpublished observations) based on recombination frequencies as illustrated by Nachman (2002) in humans (where recombination is generally expected to be suppressed near centromeres and elevated near telomeres); in the case of LG1, centromere was proposed to be towards the beginning of the group and for LG3 towards the end of the group (opposite to the proposed centromere position near UNH131 in LG3 by Lee *et al.,* 2004). These observations from Dr. Thomas Kocher agreed with the findings from Chapter 3, associating LG1 with a small submetacentric chromosome and locating two markers along the large arms by FISH for its orientation, then considering the centromere somewhere at the beginning of the LG; and association of LG3 with chromosome 1, locating this time several markers along the large arms. Moreover, centromere location also based on recombination frequencies on LG6, LG7, LG10 and LG12 agreed with the centromere orientation of the physical maps proposed in chapter 3, giving enforcement to the location of the sex range on LG1, and the red skin colour/black blotches gene and marker *Oni*159 anchored on LG3.

5.5.6 Conclusions

Despite the efforts to link sex-linked AFLPs into one of the two LGs analysed, there is no conclusive evidence to genetically confirm whether these markers truly belong to LG3, LG1 or another LG.

The low marker density maps developed in the present research (main cause of the low linkage LOD scores, <3.0) didn't allow a full comparison among the different genotypes and phenotypes. Nevertheless, a putative stronger influence was detected from the genotype rather than the phenotype, with shorter LGs in XY crosses and larger in XX and phenotype influences observed within Fam.A XY with larger maps on males than females.

Discarding any environmental influences, the genetic dosage effect model is giving a new insight into the interaction effect from the different strengths of sex chromosome alleles (suppressing or determining one of the sexes). At the same time, the main sex determining locus has been confirmed in *O. niloticus*, located close to the middle of one of the small submetacentric chromosomes and tightly associated with the marker UNH995 (LG1).

O. niloticus (XY system), chromosome 1 (LG3) appears to be of autosomal influence on sex determination with a minor penetrance and with the capacity to influence a switch of sex in both directions and with different magnitides upon the family background. Moreover, the epistatic interaction between LG1 and LG3 alleles observed in *O. aureus* (Lee *et al.,* 2004) should be responsible for some of the minor disequilibriums observed in sex ratios. In the case of *O. aureus* (WZ system), opposite to *O. niloticus*, the small

submetacentric chromosome associated with LG1 seems to play one of the autosomal sex influence roles (Lee *et al.,* 2004).

As proposed by Lee *et al.* (2003; 2004) in *O. aureus* on W-chromosome alleles, it would be necessary to quantify the strength of different Y-chromosome alleles in *O. niloticus*, such as 258bp from UNH995, from different individuals, strains and species, and select genotypes by marker-assisted selection (MAS) that generate a higher percentage of males for commercial production.

On LG3, the new polymorphic marker *Oni*159 (associated with dmrt1 gene from chapter 4) was anchored to the first part of the linkage group by its linkage to GM354.

Red skin colour/black blotches locus has been identified close to the centromere on LG3. Its location is proposed to be linked to a highly recombinant spot (hot spot). Flanking alleles associated to this locus have been identified from the markers GM128 (95bp allele) and GM526 (240bp allele), highly predicting the presence of the recessive red locus (r) that causes black blotching on the skin.

Further investigation of tightly linked alleles to the red skin colour/black blotches locus (mapping the markers UNH108 and GM567) will be necessary for a more precise prediction of this QTL and subsequently for marker-assisted selection.

With the information presented from LG1 and 3 in *O. niloticus*, along with their respective physical maps, studies for the identification of synteny with

model organisms such as zebrafish, pufferfish, catfish, etc. or other Lake Malawi cichlids would be of great advantage for the evolutionary and chromosome dynamics understanding of this species.

Chapter 6 GENERAL DISCUSSION

6.1 Integration of physical and linkage mapping

The main sex determination locus in *O. niloticus* which has an XY/XX system is on a small submetacentric chromosome (LG1) (Lee and Kocher, 2007; Lee *et al.,* 2003) which has been confirmed in the present research with different families. In *O. aureus,* with a WZ/ZZ system the main sex determination locus has been localised to chromosome 1 (LG3) with an apparent epistatic interaction with a small submetacentric chromosome (LG1; Lee *et al.*, 2004). The dominance of WZ system over the XY system has also been demonstrated in the *Oreochromis* F2 hybrid (red XX neomale *O. niloticus* x normal coloured ZZ neofemale *O. aureus*) (Lee *et al.,* 2004; Lee *et al.,* 2005), and the F2 hybrid from *O. mossambicus* and *O. aureus* (Cnaani *et al.,* 2004).

From the current study, a detailed analysis of the chromosome 1 physical map in *O. niloticus* demonstrated differences in recombination rate along the q arm compared to LG3 distribution. These differences indicate the localisation of at least one hot spot (highly recombinant loci) on the long arm close to the centromere (approximately 5 cM apart) and suppression of recombination on the distal half of the same arm nearer the telomeric region. This area is associated with: 1) The origin of the unpaired bivalent in XY genotypes during early pachytene (Carrasco *et al.,* 1999), 2) the largest accumulation of heterochromatin and repetitive sequences observed in the chromosomes of this species (Harvey *et al.,* 2003b; Oliveira and Wright, 1998; Oliveira *et al.,* 2003), and 3) cross hybridisation differences between XX and YY fish (Harvey *et al.,* 2002b).

Orientating LG3 with respect to chromosome 1, the centromere is proposed to be close to the marker GM526, leaving tentatively only the markers GM385 and UNH982 on the short arm (p arm) whilst the rest of the linked markers belong to the larger arm (q arm) of the same chromosome. Centromere localisation on LG3 was possible considering that meiotic observations are a better representation of the LGs distribution (Cheng *et al.,* 2001; Peterson *et al.,* 1999; Wallace and Wallace, 2003) and allowing physical distances (Flpter) to be proportionally calculated in recombination distances (cM). At the same time, it was observed that LG3 is still missing at least 20% of the total length of chromosome 1 basically due to the localisation of the marker GM354 (0cM) at Flpter 0.8 on the large arm.

The newly discovered polymorphic marker *Oni*159 that belongs to the BAC clone containing a copy of the dmrt1 gene has been confirmed to belong to LG3 and is closely linked to marker GM354. Boonphakdee (2005) previously identified dmrt1,2,3 cluster (Flpter 0.57) and dmrt1 gene (Flpter 0.85) on chromosome 1 by FISH. Considering that another copy of the dmrt1 gene has been linked to LG12 (Lee *et al.,* 2005) and confirmed by FISH in this research, it would be necessary to compare sequences of dmrt1 at these three different locations before concluding whether this represent another example of a duplicate gene. Such duplicated gene have been observed with: 1) two different locations of the same microsatellite locus (UNH111; Palti *et al.*, 2001), 2) Gene duplication of Wt1 (Lee and Kocher, 2007) showing greater coding-sequence similarity in Nile tilapia or a modified translocated gene copy, and 3) the apparent duplication of insulin gene associated to LG3 and LG10 as observed in the present research.

The physical map of LG1 orientates the centromere at the beginning of the LG, close to the ovarian cytochrome P450 aromatase (CyP19A1) gene. Moreover, an inversion of telomeric markers on the large arm was detected when comparing the *Oreochromis* F1 hybrid map (Lee *et al.,* 2005) and the *O. niloticus* map. This inversion is suggested to be indicative of chromosome instability or a simple rearrangement between species as suggested by Agresti *et al.* (2000) and Lee (unpublished observations), causing loci to be linked in different orders. At this stage it is still unclear if this inversion would be related to sex differences, given that a more complex rearrangement has been observed specifically on LG9 when comparing *O. niloticus* (Kocher *et al.,* 1998) and *O. aureus* (McConnell *et al.,* 2000). In contrast, the order of markers on LG3 seems to be well conserved.

6.2 Putative sex-determination and red skin colour chromosomes in Nile tilapia

Several lines of evidence support LG1 and 3 as the strongest candidates in sex determination. These include 1) a general suppression in recombination on the larger chromosome, giving shorter LG relative to the groups mapped on the small chromosomes; 2) significant differences in recombination observed in *O. niloticus* and *O. aureus* between males and females when comparing LG1 and LG3 (Lee *et al.,* 2004); Dr. Bo-Young Lee, unpublished observations) and 3) two pair of bivalents unpaired during early pachytene showed in *O. aureus* (Campos-Ramos *et al.,* 2001). Despite previous evidence pointing clearly to chromosome 1 as the main sex determining chromosome in Nile tilapia, physical mapping by FISH techniques of several markers from LG1

demonstrated the different location of this group on a small submetacentric chromosome and placed LG3 on chromosome 1. This small submetacentric chromosome (34% of the total length of chromosome 1) in *O. niloticus* is proposed to represent chromosome 4, 5 or 7 in the karyotype of Martins *et al.* (2004), an issue that would be possible to clarify with a dual physical hybridisation of SATA/SATB repeats and the BAC clone containing the marker UNH995.

In the present research it has been demonstrated that the main sex determination locus in Nile tilapia is close to the middle of the larger arm of a small submetacentric chromosome (LG1). However, a previous study (Karayucel *et al.*, 2004) failed to find a statistical association between sex and LG1. Instead, they noted an autosomal influence with minor penetrance in sex determination associated with the red skin colour locus on the long arm of chromosome 1 (LG3). The partial penetrance in sex from a locus on chromosome 1 is supported with the presence of the gene responsible of the red and black blotches skin colour, close to the centromere on the long arm.

When comparing *O. niloticus* and *O. aureus* the master sex-gene seems to switch between LG1 and LG3. When the main sex-gene is situated on LG1 (in *O. niloticus*) it is localised between Wt1b and UNH995 markers (within 5.9 cM gap) (Lee and Kocher, 2007) and in *O. aureus* and its hybrids (crossed with *O. niloticus* or *O. mossambicus*), the main sex-gene was demonstrated to lie on LG3 between GM354 and GM271 (within a 13 cM gap) (Cnaani *et al.,* 2004; Lee *et al.,* 2005). The evidence of Lee *et al.* (2005) concerning the epistatic interaction between LG1 and 3, and the mapping of sex on LG1 and the red

skin colour/black blotches loci on LG3 previously associated with autosomal influence (Karayucel *et al.,* 2004) clearly demonstrates that whenever one locus is acting as a dominant sex-determining gene, the other acts as an epistatic locus of low penetrance. This effect has been observed in other fish species such as stickleback *Gasterosteus* (Peichel *et al.,* 2004) and medaka *Oryzias* (Volff *et al.,* 2007), where there is evolutionary instability of sex-determination systems even among closely related species. Moreover, in *Oreochromis* species an autosomal effect has been demonstrated with the capacity to influence sex reversion in both directions (Avtalion and Don, 1990; Desprez *et al.,* 2006; Ezaz *et al.,* 2004c; Shirak *et al.,* 2002).

Despite these lines of evidence supporting LG1 and LG3 in sex determination, it is important to remark that in some of the *O. niloticus* families studied in this and previous research sex linkage was not shown with LG1 or LG3 (Lee *et al.,* 2003), leaving open the possibility of an alternative, third LG participating in sex determination. At this stage, observations made by Palti *et al.* (2002) and Shirak *et al.* (2002, 2006) detecting QTLs for sex-specific mortality on LG2 (UNH159), LG6 (UNH231) and LG23 (UNH848 and UNH879), and a QTL for sex-determination on LG23 (UNH216) could answer some of the questions arising from families with no apparent sex linkage to LG1 and 3. In these cases, LG23 is the third most likely candidate due to the recent mapping of two sex-related genes, Anti-Mullerian hormone (*Amh*) and Dmrta2, associated with sex determination and sex-specific mortality respectively (Shirak *et al.,* 2006).

6.3 Comparison with close related species

A rearrangement of the three pair of chromosomes from the *O. karongae* karyotype (2n=38) has been detected when compared with other seven tilapia species from the genera *Oreochromis*, *Tilapia* and *Sarotherodon* (2n=44), reducing chromosome numbers by fusions of three small pair of chromosomes. Interstitial SATA *in situ* hybridisations proved to be very useful in detecting chromosome fusions by recognition of ancient centromeric repetitive sequences (Martins *et al.,* 2004). It was then concluded that there had been a telomere-telomere fusion in chromosome 2 (subtelocentric), a centromere-telomere fusion in chromosome 3 (subtelocentric) and a centromere-telomere fusion in chromosome 4 (submetacentric). A Robertsonian translocation on chromosome 4 is still in question as proposed by previous authors (Harvey *et al.,* 2002a) due to a clear gap observed between the interstitial SATA signal and the centromere.

The assignment of LG1 and LG3 to a small submetacentric chromosome and chromosome 1 respectively, was well conserved among the different species studied, except for *O. karongae* that locates LG1 on the subtelocentric chromosome 2. Nevertheless, specific karyotypic differences were observed among the other six species studied (*O. aureus, O. mossambicus, O. mortimeri, S. galilaeus, T. rendalli, T. zillii*), reflected in the different number of subtelocentric/acrocentric and metacentric/submetacentric chromosomes, giving another insight into the evolution of these relatively close related tilapia species (Kocher, 2004; Turner *et al.,* 2001a).

The development of two novel meiotic karyotypes from *O. niloticus* and *O. karongae,* based on the real DAPI stain picture and straightened chromosomes for an accurate measurement of total chromosomal length and centromere positions, allowed the creation of a precise record of relative distances for the categorisation of the whole set of chromosomes. They also facilitate further comparative karyotype studies among close related species. Despite these improvements, assignment of specific tilapia LGs to chromosomes in combination with SATA probes will be crucial for the differentiation of very similar chromosomes, especially for the smaller sizes.

6.4 Assignment of some autosomal linkage groups

The relatively similar sizes of small chromosomes in *O. niloticus* is reflected in the similar LG length as observed among LG1, LG6, LG10 and LG12 assigned in this research, ranging from 50 (LG10) to 65 cM (LG12). Nevertheless, when comparing LG7, associated to chromosome 2 and also with 65 cM (and despite having LG16-LG21 (Shirak *et al.*, 2006), LG4 and LG11 with 77cM or more), indicates in general terms that recombination is enhanced in small chromosomes and suppressed in the larger chromosomes. A similar phenomenon has been observed in zebrafish following full assignment of LGs to chromosomes (Phillips *et al.,* 2006a). This increment in recombination on small chromosomes is thought to occur ensuring one crossover per chromosome arm.

6.5 Sex-determination model

It has been demonstrated that different allelic combinations on the sex chromosomes from parents lead to different sex ratios in the progeny. Even alleles from the same locus showed different levels of strength towards male or female determination as observed in XY animals with 194bp and 235bp alleles respectively or 185bp and 258bp in the tightly linked UNH995, giving a higher proportion of males in the first case by the presence of the Y allele linked to the 235bp allele from UNH995 even with the combination of the X allele linked to 185bp allele. Also, It has been detected different strengths towards females of the X allele 185bp coming from different broodstock.

Comparing with tilapia WZ systems and WZ/XY hybrids, the proposed sex-determination model in tilapia extends to the interaction of alleles not only from the small submetacentric chromosome (LG1) but also from chromosome 1 (LG3) where XY-ZZ hybrid produce 100% males by the sum of effects from Y and the two Z, and XX-WZ hybrids are 100% females by the sum of effects from W and the two X. XY-WZ hybrids also produce 100% females with a dominant influence from the W chromosome (master female gene or male suppressor) in front of Y chromosome (master male gene).

An important aspect that still has to be revealed is the reason why sex-linkage is only present in families segregating the allele 235bp from UNH995 (four out of six families) on LG1, while in the others, sex linkage did not appear to be associated with either LG1 or LG3 (possibly getting stronger sex determination influence from LG23 as mention in section 6.2). This phenomenon was also observed by Lee *et al.* (2003) when one of three *O.*

niloticus families analysed did not show linkage to LG1 at all, suggesting further evidence of additional genetic and/or environmental factors regulating sex determination in some families. The same author recognised that different Y chromosome alleles have different strength or penetrance on sex determination. In light of the findings in the present research (keeping the same environmental factors), it leads to the consideration of a model where a number of genetic factors interact positively or negatively in favour of one sex at the top of the main sex determination cascade.

As mentioned by Mittwoch (2006), Y or W chromosomes, minimise the effect of different variables that affect the sex-development of a heterozygous embryo, resembling in that respect the effect of a single gene even when there are other several factors (genetic and environmental) that could affect the sex-determination in early stages. Moreover, a gene dosage-dependent model of sex determination has been proposed in birds, with the effect of two allelic copies of dmrt1 ZZ containing copies of the dmrt1 gene (Clinton and Haines, 1999), or in the X, Y, W system in the platyfish *Xiphophorus maculatus* where the higher copy number and expression of the male regulator in XY and YY lead to the male phenotype (Volff *et al.,* 2007).

6.6 Evolution of sex-determination systems in tilapia

Cichlids and many other fishes studied have been shown to have young and independent sex differentiating systems that evolved within the last 10 million years, so that for instance most of the YY genotypes are fertile and viable in contrast to mammals, indicating low Y chromosome degeneration (Volff *et al.,* 2007).

In tilapia, all the evidence to date strongly indicates a system in transition that is moving from WZ heterogametic female to XY heterogametic male in *O. niloticus,* with a male determining locus on the Y chromosome (LG1); and a clear interaction of both systems in *O. aureus* but probably moving towards WZ with a major female W locus (LG3). This species also shows unpairing in a small bivalent during SC observations. This transitory and probably drifting system is expressed graphically in Figure 6.1. Due to the apparent dominance of W chromosome over Y chromosome in hybrid crosses, it is proposed that tilapia have an ancestral WZ- and a nascent XY-system. *O. niloticus* and *O. aureus* represent at the same time an example of two systems that were generated from two different autosomal regions and with a common ancestor, which may have had temperature-dependent sex determination as observed in reptiles (Ezaz *et al.,* 2006). Different master sex regulators have been described between species within the same genera of other organisms, e.g. *Oryzias*, *Gasterosteus, Xiphophorus* and other poeciliids (reviewed in: Volff *et al.,* 2007). Nevertheless, further work need to be done to confirm these different findings in *O. niloticus* and *O. aureus*, e.g. 1) a detailed FISH comparison of the small chromosome bivalent that shows SCs in *O. aureus* but not in *O. niloticus*, and 2) an analysis of the grade of accumulation of repetitive

sequences close to the sex determination locus have to be undertaken for a better definition of the sex-drift direction.

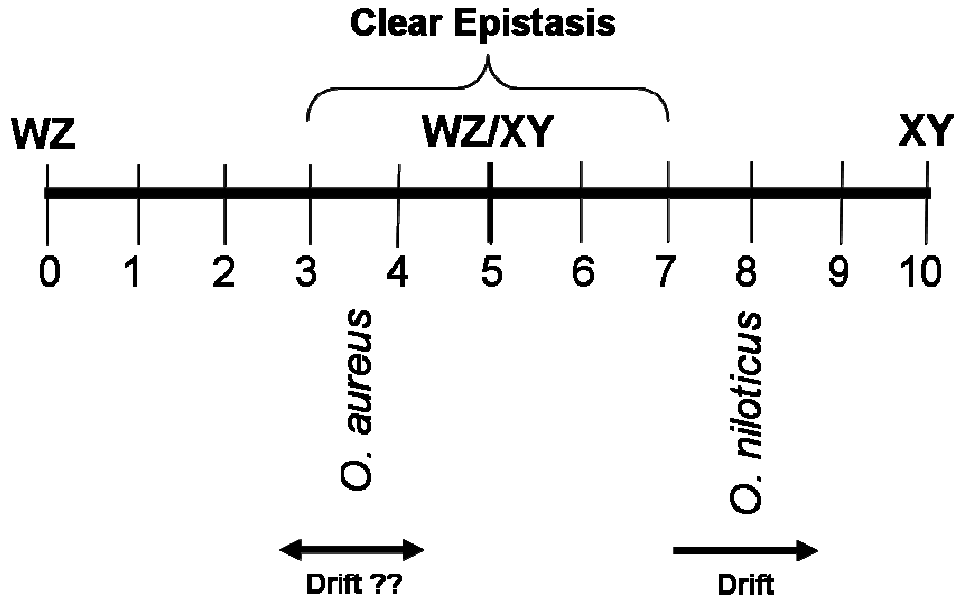

Figure 6.1 Graphical representation of the WZ/XY sex-transition or -drift systems observed in *Oreochromis niloticus* and *O. aureus*. The scale positions for each of the species represent a suggestion according to the observed genetic and physical characteristics from sex chromosomes counting towards sex determination such as sex-linked locus, allelic strengths, unpairing of bivalents, heterochromatin blocks and repetitive sequences, progeny sex ratios and hybrid crosses . Scale 0-10 was set arbitrarily.

This transitory stage of sex determination in *O. niloticus* moving from chromosome 1 (LG3) to the small submetacentric chromosome (LG1) is firmly suggested by the evidence from chromosome 1 that shows typical characteristics of sex chromosomes around the sex determination locus such as heterochromatin clusters with the presence of repetitive sequences, transposable elements and trans-chromosomal duplication of genes confirmed in the present research. Heterochromatin accumulation had been also observed in *O. latipes* (Kondo *et al.,* 2006) and *X. maculatus* (Nanda *et al.,* 2000) as clear

examples. However, in *O. niloticus* the linkage analysis showed sex determination locus to be located on LG1.

The observation of a dmrt1 gene copy by physical and linkage analysis on chromosome 1 (the other copy is located in a small subtelocentric chromosome, LG12), supports the idea of a WZ system on these chromosomes and their role in sex-determination or -differentiation. In birds dmrt1 has been observed on the Z but not in the W chromosome making it an excellent candidate for a Z-linked male sex-determining gene (Ferguson-Smith, 2007). Furthermore, as mentioned in the previous section, higher levels of dmrt1 expression have been confirmed in ZZ males than ZW females prior to and during the critical period of sex differentiation in the gonads of chicken embryos (Clinton and Haines, 1999). In another example, a copy variant of the same gene, dmrt1bY, in male medaka has been demonstrated for the first time in non-mammalian vertebrates to have a role as a master sex-determining gene (Matsuda *et al.,* 2002). It shows an early expression during embryonic development before gonad formation whilst the autosomal dmrt1 is expressed later in spermatogonial differentiation (Kobayashi *et al.,* 2004; Hattori *et al.,* 2007). Thus, it could be suggested that dmrt1 or a variant of this gene on chromosome 1 has a key role in the WZ system in *O. aureus*, and even with a low penetrance, in XY sex determination in *O. niloticus* by a balance of dmrt1 dosage on Z, and Y and W-linked sex determination factors.

In support of all the above observations and based on comparative genomics of sex determination systems from several vertebrate species, Volff *et al.* (2007) suggest an evolutionary model of sex-chromosomes with sex-

determination mechanisms in constant change. In this model, the gradual degeneration of the Y or W chromosome reaches a point of a possible haploinsufficiency and a consequent reduction of fitness, leading to their replacement by new autosomes with a new or duplicated sex determining gene. Ferguson-Smith (2007) would add to this scenario that failure of an XY or WZ system also results in X0 or Z0 system as reported in some fish species (Tave, 1993). Finally, it has to be added that not only genetic but also environmental influences represent a very important selection pressure in driving the genome towards the most successful sex-determination system.

6.7 Further directions

The development of a more accurate and integrated physical-genetic map and the novel meiotic karyotypes leave a solid base for the location and integration of new markers and genes, and a platform where more accurate comparisons and chromosome changes could be detected among different tilapia species. So the full assignment of linkage groups to chromosomes will be of great advantage for the characterisation and management of specific loci such as the major sex-determination locus.

Further studies on LG23 should be considered a priority such as physical mapping and testing for the identification of a putative sex-determination locus previously suggested on this LG (Shirak *et al.,* 2006) by analysing the six different F1 parents from Fam.A already tested for LG1 and 3. For the application of FISH techniques, the identification of positive BAC clones containing the markers UNH848, UNH216 and UNH879 used as probes will be

essential along with the genotyping of Fam.A as mentioned above in order to check if any minor penetrance of sex-determination came from this potential autosomal LG in Nile tilapia.

Due to the clear recognition of LG1 as one of the main players in sex determination, it will be relevant to look for tightly sex linked markers in the interval between GM201 or WT1b and UNH995 markers, and then “walking” towards the sex locus with the aid of the BAC-based physical map available for tilapia (http://hcgs.unh.edu/cichlid/). However, there are major obstacles to overcome, such as the long distance (approximately 2.5 cM gap between WT1b and sex locus) and the high frequency of repetitive sequences close to the sex locus.

At the same time, homologous sequences from chromosome 1 found in the cichlid *Astatotilapia burtoni*, the stickleback *Gasterosteus aculeatus* and zebrafish *Danio rerio* could serve as a starting point for a more detailed comparative genomics analysis to understand widely the evolution of this chromosome towards sex determination and synteny with other genomes.

As was already demonstrated with the present fish crosses, a search for the identification of tightly sex-linked alleles in the commercially important tilapia species will have a direct application in broodstock management, eliminating the tedious process of progeny-testing, and moreover, selecting in favour of the strongest Y and/or Z chromosome alleles.

The dmrt1 copy gene on chromosome 1 needs to be compared by sequencing with the copy found on a small subtelocentric chromosome (LG12) in order to explore the extension of homology between these two genes.

Finally, microdissection and fish painting of sex chromosomes on *O. karongae* as well as a hybrid *O. niloticus* X *O. karongae* will help to gain a better understanding of the dynamic sex-determination system due to their differences in chromosome number and a chromosome fusion in O*. karongae* involving the main sex-chromosome target in *O. niloticus*.

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APPENDICES

Appendix I. Formulation of Solutions and Buffers

-General Use-

- **Methicillin and Ampicillin stock solution**: 80 mg ml^{-1} of Methicillin and 20 mg ml^{-1} in molecular grade autoclaved water; store at -20°C.
- **Chloramphenicol stock solution**: 34 mg ml^{-1} in absolute ethanol; store at -20°C.
- **SOC Medium**: 2% Tryptone, 0.5% Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM glucose.
- **IPTG (isopropyl β-D-thiogalactopyranoside) stock solution**: To prepare 100 mM stock solution. 238 mg IPTG in 10 ml molecular grade water. Filter-sterilise (0.2 µm); store at -20°C.
- **X-gal (5-bromo-4-chloro-3-indoyl-β-galactopyranoside) stock solution**: To prepare 40 mg ml^{-1} stock solution. 400 mg X-Gal in 10 ml dimethylformamide in a brown bottle; store at -20°C.
- **Loading Dye II 6×**: 15% Ficoll in molecular grade water, 0.25% Xylene Cyanol FF, 0.25% Bromophenol blue. Keep at room temperature.
- **TE**: 10 mM Tris-HCl, 1 mM EDTA; pH 8.0.
- **TAE buffer 50×**: 242 g Tris-HCl, 57.1 ml Glacial Acetic Acid, 100 ml 0.5 M EDTA; pH 8.0. Bring to 1l with molecular grade water.
- **TBE buffer 10×**: 108 g Tris-HCl, 55 g Boric Acid, 8.3 g EDTA (Na_2), bring to 1l with molecular grade water. Filter with 0.5 µm whatman filter.

-Solutions to fix cell colonies onto Hybond–N+ membrane-

- **Lysis or SDS (Sodium Lauryl Sulfate) stock solution**: 10% SDS (in molecular biology grade water). Heat to 68°C, do not autoclave. pH 7.2.
- **SSC (Sodium Saline Citrate) stock 20×**: 3 M NaCl, 0.3 M Sodium Citrate; pH 7.0. Autoclave.
- **Denaturing solution**: 0.4 M NaOH, 0.6 M NaCl. Autoclave.
- **Neutralising solution**: 0.5 M Tris-HCl, 1.5 M NaCl; pH 7.5. Autoclave.
- **Conditioning and Digestion solution**: 2× SSC from the 20× SSC stock solution described above.
- **Rinsing solution**: 2×SSC, 0.1% SDS. Autoclave.
- **Hybridisation (Church's) solution** (Church and Gilbert, 1984): 0.5M phosphate buffer, pH7.2; 7% (w/v) SDS, 10mM EDTA. Store at -20°C.
- **Pre-Hybridisation solution**: 50× Church's solution, 10% SDS, 20× SSC. pH 7.7. Do not filter, store at 4°C.
- **Washing solution**: Low stringency - 6× SSC, 0.2% SDS. Medium stringency – 5× SSC, 0.2% SDS.

-Solutions for FISH technique-

- **Denaturing solution**: 70% formamide, 2× SSC.
- **Washing solution**: 50% formamide, 2× SSC.
- **Pre-hybridisation solution**: To prepare 150ml. 4× SSC, 75 µl Tween20 solution.
- **Hybridisation solution**: Pre-hybridisation solution, 60µl of 25% BSA.
- **Detection solution**: For two slides in dual hybridisation. 1.7 µl Cy3-streptavidin, 38.3 µl anti-digoxygenin, 20µl 25% BSA, 440 µl Pre-hybridisation solution.

Appendix II. Preparation and Application of Acetocarmine Staining (1%)

According to Guerrero and Shelton (1974)

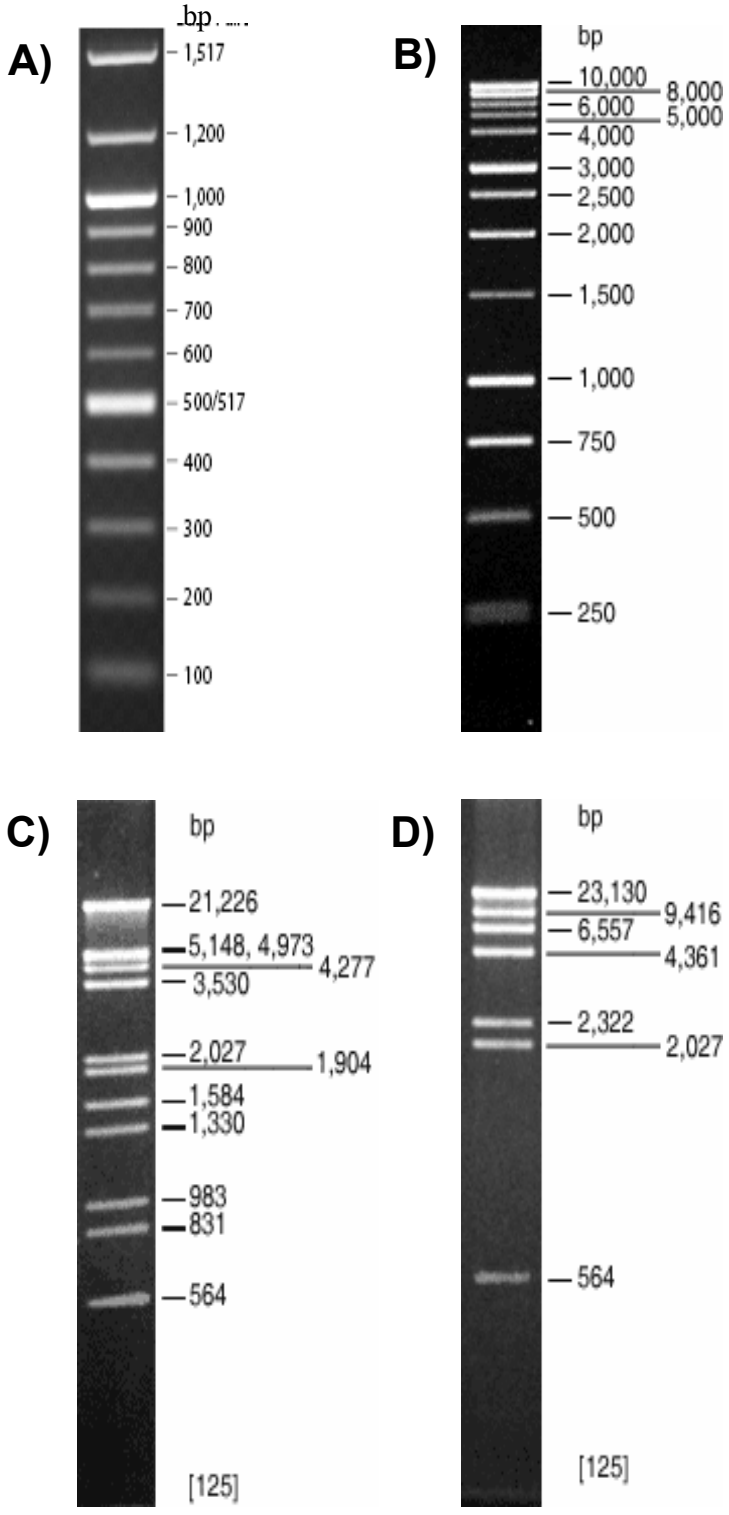
45% Glacial acetic acid 100 ml

Carmine 0.5 g

Boil acetic acid and carmine gently for 5 minutes in a beaker covered with a watch glass. Do this in a fume hood. Shake while the mixture cools. Filter cooled solution through #2 Whatman paper. Filtration takes a long time and several changes of paper are usually necessary. Store stain in brown glass at room temperature.

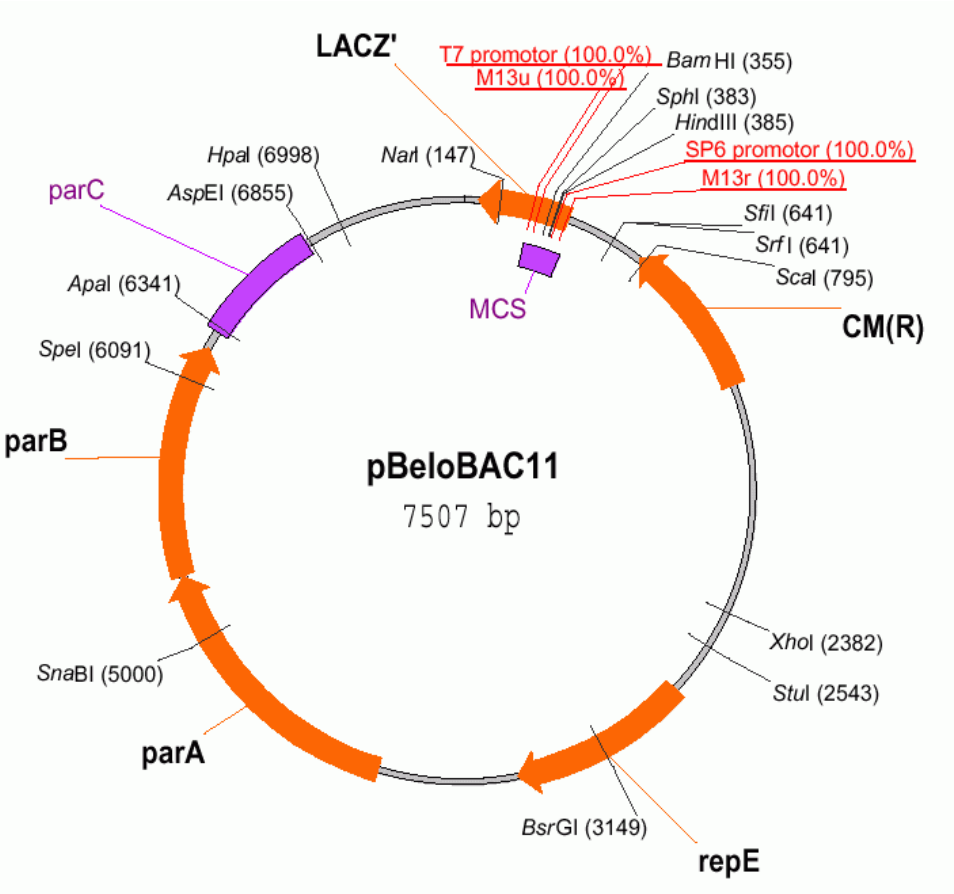
Fresh or preserved material is transferred into 1 % aceto-carmine for at least 30 min and then analysed by the squash method. If the material was fixed for a longer time, it requires a longer staining time (up to several days) to reach good contrast. If the material is to be analysed immediately, fix and stain the tissue in one step using the 1 % aceto-carmine solution.

Appendix III. Standard DNA markers



Standard DNA ladders showing marker bands generated from 0.5µg loaded on 1.3% TAE Agarose gel: A) 100bp from New England BioLabs, UK; B) 1Kb, C) λDNA *Ecor* I and *Hind*III, and D) λDNA/*Hind*III ladders from ABgene labs, UK.

V-pBeloBAC11 DNA: Restriction map

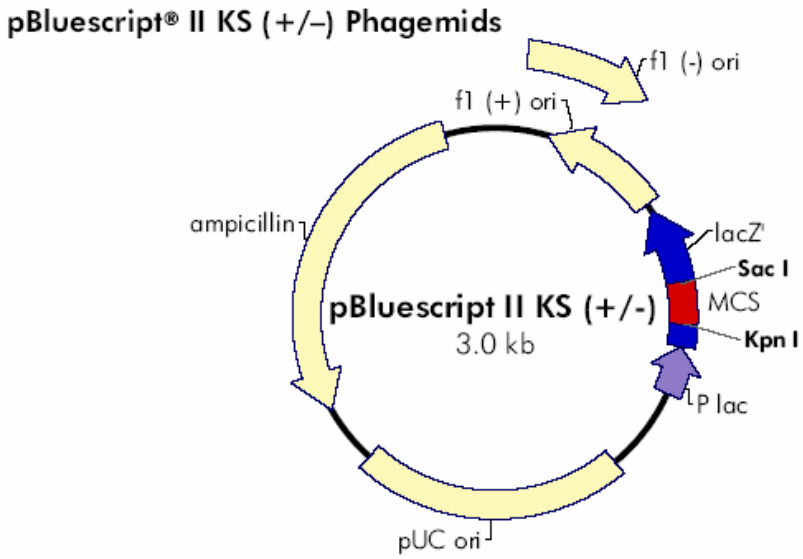


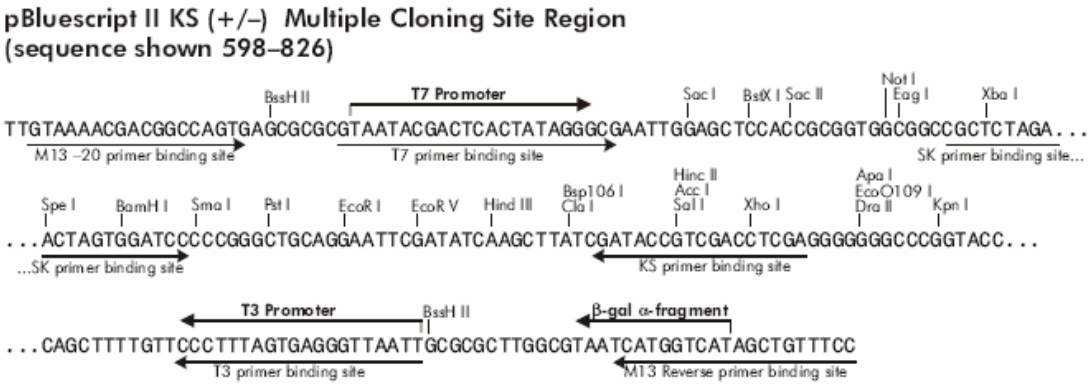
Feature	Coordinates (bp)	Source
T7 promoter	312-329	T7
SP6 promoter	398-414	SP6
lacZ	56-436	-
cat (CmR)	766-1425	Tn9
redF fragment	1644-1991	F
repE (repA)	2765-3520	F
Ori2 (OriS)	2370-2436	F
sopA (parA)	4108-5274	F
sopB (parB)	5274-6245	F
sopC (parC, incD)	6318-6791	F
cos site	7050-7449	lambda
loxP site	7467-7500	P1

Ori= origin of replication
Cm= chloramphenicol

pBeloBAC11 DNA (7507 bp) showing the restriction map and bp position of each composing fragment. Source: RZPD Deutsches Ressourcenzentrum für Genomforschung, Germany.

Appendix IV. pBluescript II KS- Clonning Vector map





Feature	Nucleotide Position
f1 (+) origin of ss-DNA replication [pBluescript KS (+) only]	135–441
f1 (–) origin of ss-DNA replication [pBluescript KS (–) only]	21–327
β-galactosidase α-fragment coding sequence (lacZ')	460–816
multiple cloning site	653–760
T7 promoter transcription initiation site	643
T3 promoter transcription initiation site	774
lac promoter	817–938
pUC origin of replication	1158–1825
ampicillin resistance (*bla*) ORF	1976–2833

pBluescript®II KS (+/-) Phagemid Vector (3 Kb) showing the sequence from 598 to 826bp with restriction sites and features according to nucleotide position. Genbank accession numbers: X52327 (KS+) and X52329 (KS-). Source: Stratagene, USA.

Appendix V. Tilapia BAC library clones used for physical mapping and contig related

SEX LINKED MARKERS AND GENES (Green tape)					PROJECT and CONTIG RELATED. Version 6.0, Last Update: 22 May 2003												
No.	Well ID	BAC ID	Marker	LG	Tilapia 05k t5 1e-08	Tilapia 10k t5 1e-08	Tilapia 15k t5 1e-08	Tilapia 20k t5 1e-08	Tilapia 25k t5 1e-08	Tilapia 30k t5 1e-08	Tilapia t3 1e-08	Tilapia t5 1e-06	Tilapia t5 1e-07	Tilapia t5 1e-08	Tilapia t5 1e-09	Tilapia t5 1e-10	Tilapia t7 1e-08
1	A01	b04TI074AA04	GM271	3	0	0	0	3086	2958	3064	2733	2240	2636	3031	3376	0	3426
2	B01	b04TI056BC05	GM271	3	?												
3	C01	b03TI070BF03	GM204 *	3	0	2026	0	1509	2290	0	1762	1688	1953	2200	0	0	0
4	D01	b04TI071BH02	GM204 *	3	128	613	728	0	1330	2184	1762	1318	1498	1670	1891	2005	2078
5	E01	b03TI062CG03	GM354	3	0	0	0	0	2829	2948	2609	2185	2565	2945	3277	3605	3721
6	F01	b03TI066DH01	GM354	3	0	0	0	0	2381	2556	2479	1956	2298	2608	2870	3143	2757
7	G01	b04TI004DB08	GM354	3	?												
8	H01	b04TI004AA02	DMT	12	0	1254	0	0	1667	1909	1458	1576	1818	2036	2291	2487	2385
9	A02	b03TI082DB05	DMO		0	0	0	0	445	115	190	195	208	207	222	220	142
10	B02	b04TI055AC04	DMO		0	597	1056	206	445	115	190	195	208	207	222	220	142
11	C02	b04TI076CC06	CLCn5	3	0	0	1962	1971	0	1984	1385	1363	1603	1786	2890	3162	3318
12	D02	b04TI060DC04	Wt1a	7	0	0	1005	0	0	1679	1214	179	1574	1819	2047	2197	2259
13	E02	b04TI057DA02	Aromatase (ovary)		11	0	0	0	105	39	72	4	52	50	50	53	53
14	F02	b04TI056AD04	GM180	3	0	0	2299	2285	0	1578	1106	1048	1170	1286	1366	1434	1500
15	G02	b04TI071CH12	UNH115	3	0	1266	0	0	0	109	183	185	197	198	200	198	87
16	H02	b03TI066CH03	Insulin	10				1040	2283	2462	1986	1365	1557	1800	1933	2064	1882
17	A03	b03TI094DB09	UNH995 & UNH104	1	0	0	0	0	1669	2026	1196	965	1072	1179	1329	2512	1163
18	B03	b04TI071CD06	UNH995 & UNH104	1	0	1069	453	0	1669	1164	118	159	178	185	189	203	401
19	C03	b04TI025AC01	UNH995 & UNH104	1	0	0	292	0	707	1035	1196	965	1072	1179	1373	1541	1163
20	D03	b04TI035BB11	UNH995 & UNH104	1	162	0	0	0	2926	1865	1633	965	1769	1992	2158	2324	2243
21	E03	b03TI085CC01	UNH868	1	0	0	721	0	406	1020	791	533	608	643	674	732	433
22	F03	b03TI085CE09	UNH868	1	0	0	0	3417	0	20	28	23	25	26	26	0	0
23	G03	b03TI093DA01	UNH918	6	0	0	0	0	0	3307	1695	1411	1619	1809	1942	2204	2361
24	H03	b03TI077DC09	UNH948	6	0	1166	0	0	3169	3263	2542	2167	2548	2914	3237	3559	3389

* = Not available sequence; Green tape= refers to the colour of the label used on the vial containing each BAC clone.

Appendix VI. (Continued) Tilapia BAC library clones used for physical mapping and contig related

RED SKIN COLOUR RELATED (Blue tape)					PROJECT and CONTIG RELATED. Version 6.0, Last Update: 22 May 2003												
No.	**Well ID**	**BAC ID**	**Marker**	**LG**	Tilapia 05k t5 1e-08	Tilapia 10k t5 1e-08	Tilapia 15k t5 1e-08	Tilapia 20k t5 1e-08	Tilapia 25k t5 1e-08	Tilapia 30k t5 1e-08	Tilapia t3 1e-08	Tilapia t5 1e-06	Tilapia t5 1e-07	Tilapia t5 1e-08	Tilapia t5 1e-09	Tilapia t5 1e-10	Tilapia t7 1e-08
25	N/A	b04TI053CE04	Trp1 *	3	0	0	0	1570	0	1123	252	316	350	360	386	385	471
26	N/A	b04TI051BF05	GM128	3	0	0	0	0	0	3268	2916	2280	2696	3202	3577	3963	3665
27	N/A	b04TI007BH12	GM128	3	0	0	0	0	0	3268	2916	2280	2696	3202	3577	3963	3665
28	N/A	b04TI055DC08	GM150	3	0	0	0	0	94	108	151	100	106	106	104	114	354
29	N/A	b04TI053DE10	putative Trp1 pseudogene *	3	617	0	0	359	941	768	141	294	313	953	1062	1096	687
30	N/A	b03TI073AG01	Trp1 *	3	0	2002	2811	1570	0	1123	252	316	350	360	1746	0	471
INSULIN RELATED																	
No.	**Well ID**	**BAC ID**	**Marker**	**LG**													
31	N/A	b03TI081DB12	GM561	10	480	0	0	0	1626	780	38	757	856	965	1014	1116	1002
32	N/A	b03TI070DG02	UNH915	10	0	0	0	0	0	0	584	661	739	835	878	0	0

* = Not available sequence; Blue tape= refers to the colour of the label used on the vial containing each BAC clone.

Appendix VI. FISH data from O. niloticus physical map, using probes prepared by DOP-PCR techniques

O. nilotucus FISH (DOP-PCR)								
BAC Clone no.	**Well ID**	**Marker/Gene**	**LG**	**Physical Map**	**Chrom. Type (Mitosis) & centromere FIpter**	**Comments**	**Mean FIpter (±SD)**	**Total Measures**
2T	B01	GM271	3	Chrom. 1	Subtelocentric FIpter=0.14	Strong signal, telomeres q arm	0.83 **(±0.03)**	6
				Chrom. 1	Subtelocentric FIpter=0.14	Weak signal, middle of the q arm	0.58	2
4T	D01	GM204	3	Chrom. 1	Subtelocentric FIpter=0.14	Strong signal, middle q arm	0.66 **(±0.09)**	4
6T	F01	GM354	3	Chrom. 1	Subtelocentric FIpter=0.14	Strong signal, telomeres q arm	0.80	2
7T	G01	GM354	3	Chrom. 1	Subtelocentric FIpter=0.14	Strong signal, telomeres q arm	0.76	2
8T	H01	DMT (dmrt1)	12	Chrom. 2	Subtelocentric FIpter=0.19	Weak signal, middle of the q arm	0.73	1
9T	A02	DMO(dmrt4)	3	Chrom. 1	Subtelocentric FIpter=0.14	Medium signal, near centromere q arm	0.25	2
10T	B02	DMO(dmrt4)	3	Chrom. 1	Subtelocentric FIpter=0.14	Strong signal, near centromere q arm	0.24	2
11T	C02	CLCn5	3	Chrom. 1	Subtelocentric FIpter=0.14	Strong signal, dispersed towards the telomeres q arm	0.70	2
				Chrom. 1	Subtelocentric FIpter=0.14	Medium signal, between the middle and the centromere q arm. More likely	0.35	2
12T	D02	Wt1a	7	Chrom. 2	Subtelocentric FIpter=0.19	Medium signal, centromere, possible on the q arm	0.25	1
13T	E02	Aromatase (ovary, CyP19A1)	1	Chrom. 1	Subtelocentric FIpter=0.14	Medium signal, dispersed in the middle of the q arm towards telomeres	0.67	2
				Small Chrom.	Submeta-centric FIpter=0.31	Weak signal, centromere possible on the q arm. More likely	0.37	1
14T	F02	GM180	3	Chrom. 1	Subtelocentric FIpter=0.14	Medium signal, middle q arm	0.46	1
15T	G02	UNH115	3	Chrom. 1	Subtelocentric FIpter=0.14	Medium signal, telomeres q arm	0.75	2
16T	H02	Insulin	10	Small Chrom.	Subtelocentric	Medium signal, near centromere p arm. More likely	0.34	2
				Chrom. 1?	Subtelocentric FIpter=0.14	Inconsistent and dispersed signal on the q arm	0.67	2
17T	A03	UNH995 & UNH104	1	Chrom. 1	Subtelocentric FIpter=0.14	Weak signal, dispersed middle of the q arm towards the telomeres, very weak background on several autosomes. Sex related	0.64 **(±0.09)**	6
19T	C03	UNH995 & UNH104	1	Chrom. 1	Subtelocentric FIpter=0.14	Weak signal, dispersed middle of the q arm towards the telomeres, very weak background on several autosomes. Sex related	0.74	2
22T	F03	UNH868	1	Chrom. 1	Subtelocentric FIpter=0.14	Medium signal, near centromere q arm	0.20	1
23T	G03	UNH918	6	Chrom. 1	Subtelocentric FIpter=0.14	Weak signal, middle of the q arm	0.64	2
24T	H03	UNH948	6	Chrom. 1	Subtelocentric FIpter=0.14	Medium signal, middle of the q arm	0.46	1

N/A = Not Available

T = BAC clones identified by Bo-Young Lee and Aimee Howe (Univ. New Hampshire)

Appendix VII. (Continued) FISH data from O. niloticus physical map, using probes prepared by DOP-PCR techniques

***O. nilotucus* FISH (DOP-PCR)**								
BAC Clone no.	**Well ID**	**Marker/Gene**	**LG**	**Physical Map**	**Chrom. Type (Mitosis) & centromere FIpter**	**Comments**	**Mean FIpter (±SD)**	**Total Measures**
26T	N/A	GM128	3	Small Chrom.		Strong signal, between the middle and the centromere q arm. Red skin colour related	0.51	2
27T	N/A	GM128	3	Small Chrom.		Strong signal, between the middle and the centromere q arm. Red skin colour related	0.40 **(±0.12)**	3
				Chrom. 1	Subtelocentric	Medium signal, middle q arm. More likely. Red skin colour related	0.61	1
28T	N/A	GM150	3	Chrom. 1	Subtelocentric	Medium signal, dispersed close to the centromere on q arm. More likely. Red skin colour related	0.22	1
				Small Chrom.		Medium signal, dispersed close to the telomeres on q arm. Red skin colour related	0.69	1
29T	N/A	Trp1 pseudogene	3	Chrom. 1	Subtelocentric	Medium signal, near to the centromere on q arm. More likely. Red skin colour related	0.41 **(±0.18)**	4
				Small Chrom.		Medium signal, near to telomeres on q arm. Red skin colour related	0.60	2
32T	N/A	UNH915	10	Chrom. 1	Subtelocentric	Medium signal, dispersed around the middle of the q arm, more often only one chromosome with two spots. Measures from the posterior spot. Also signal on other small autosomes. Insulin related	0.68 **(±0.03)**	3
227C	72B20	*OniY*227	3	Chrom. 1	Subtelocentric	Strong signal, middle of the q arm	0.56	2

N/A = Not Available

T = BAC clones identified by Bo-Young Lee and Aimee Howe (Univ. New Hampshire)

C = BAC clones identified by Chuta Boonphakdee (Univ. Stirling)

Appendix VII. FISH data from O. niloticus physical map, using probes prepared by NICK TRANSLATION techniques

O. nilotucus FISH (Nick Translation)

BAC Clone no.	Well ID	Marker/Gene	LG	Physical Map	Chrom. Type (Mitosis) & centromere FIpter	Comments	Mean FIpter (±SD)	Total Measures
2T	B01	GM271	3	Chrom. 1	Subtelocentric FIpter=0.14	Strong signal, telomeres q arm	0.85 (**±0.04**)	10
				Chrom. 1	Subtelocentric FIpter=0.14	Weak signal, telomeres q arm	0.75 (**±0.06**)	10
6T	F01	GM354	3	Chrom. 1	Subtelocentric FIpter=0.14	Strong signal, telomeres q arm	0.81 (**±0.04**)	9
8T	H01	DMT (dmrt1)	12	Small Chrom.	Subtelocentric FIpter=0.27	Strong signal, stronger on one of them, middle q arm. Different than Lg1, 6, 10	0.85 (**±0.11**)	27
11T	C02	CLCn5	3	Chrom. 1	Subtelocentric FIpter=0.14	Strong signal on Chrom. 1, middle between centromere and 227c.	0.34 (**±0.03**)	8
12T	D02	Wt1a	7	Chrom. 2	Subtelocentric FIpter=0.19	Strong signal, very close to centromere p arm	0.13 (**±0.11**)	14
15T	G02	UNH115	3	Chrom. 1	Subtelocentric FIpter=0.14	Weak signal, middle q arm before 227C, sometimes two autosomes	0.47 (**±0.05**)	6
						Weak signal, terminal q arm, sometimes two autosomes	0.76 (**±0.05**)	14
16T	H02	Insulin	10	Small Chrom.	Subtelocentric FIpter=0.24	Strong signal, clear in one of the autosomal, telomeres q arm	0.77 (**±0.07**)	3
17T	A03	UNH995 & UNH104	1	Small Chrom.	Submeta-centric FIpter=0.31	Strong signal, middle q arm. Different than Lg6, 10, 12. Sex related	0.67 (**±0.09**)	25
22T	F03	UNH868	1	?		Failed to give specific signals		
23T	G03	UNH918	6	Small Chrom.	Subtelocentric FIpter=0.28	Medium signal, telomeres q arm. Different than Lg1, 10, 12	0.67 (**±0.00**)	3
24T	H03	UNH948	6	Small Chrom.	Subtelocentric FIpter=0.28	Strong signal, between centromere and middle q arm. Different than Lg1, 10	0.55 (**±0.12**)	6
25T	N/A	Trp1	3	Chrom. 1	Subtelocentric FIpter=0.14	Medium signal, sharper in one of them, between 227c and centromere but closer to the last. Red skin colour related	0.20 (**±0.04**)	9
26T	N/A	GM128	3	Chrom. 1	Subtelocentric FIpter=0.14	Strong signal, very close to centromere q arm. Red skin colour related	0.21 (**±0.05**)	7
28T	N/A	GM150	3	Small Chrom.		Strong signal, middle of the q arm. Red skin colour related	0.65 (**±0.08**)	9
				Chrom. 1	Subtelocentric FIpter=0.14	Weak signal off the chromosome, near centromere q arm. Only one chromosome. More likely. Red skin colour related	0.22	1
31T	N/A	GM561	10	Small Chrom.	Subtelocentric FIpter=0.24	Medium signal, very end of telomeres q arm and touching to 16T.Different than Lg1, 6, 7, 12. Insulin related	0.78	2
32T	N/A	UNH915	10	Small Chrom.	Subtelocentric FIpter=0.24	Strong signal, more in one of them, middle q arm. Different than Lg1, 6, 7, 12. Insulin related	0.60 (**±0.08**)	23

N/A = Not Available

T = BAC clones identified by Bo-Young Lee and Aimee Howe (Univ. New Hampshire)

Appendix VIII. (Continued) FISH data from O. niloticus physical map, using probes prepared by NICK TRANSLATION techniques

***O. nilotucus* FISH (Nick Translation)**								
BAC Clone no.	**Well ID**	**Marker/Gene**	**LG**	**Physical Map**	**Chrom. Type (Mitosis) & centromere FIpter**	**Comments**	**Mean FIpter (±SD)**	**Total Measures**
dmrt4C	60B8	DMO (dmrt4)	3	Chrom. 1	Subtelocentric FIpter=0.14	Medium signal, close to the centromere q arm	0.25 (**±0.05**)	13
dmrt1,2,3 C	28F22	DMT (dmrt1,2,3)	3?	?		Failed to give specific signals		
Sox9bC	5N12	Sox9b	3?	Chrom. 1	Subtelocentric FIpter=0.14	Disperse weak signal around 227C signal on chromosome 1 q arm	0.56 (**±0.07**)	8
				Small Chrom.	Subtelocentric FIpter=0.23	Medium signal, touching centromere of small autosomal q arm	0.41 (**±0.12**)	6
InsulinC	75I10	Insulin	10	Chrom. 1	Subtelocentric FIpter=0.14	Medium signal, posterior to 227C.	0.70 (**±0.10**)	8
				Small Chrom.	Subtelocentric FIpter=0.21	Weak signal in several occasions, telomeres q arm. More likely	0.79	1
227C	72B20	*OniY*227	3	Chrom. 1	Subtelocentric FIpter=0.14	Strong signal, almost middle q arm, between 11T & 15T. Sex related	0.57 (**±0.04**)	27
InsulinC	75I10	*OniY*382	3	Chrom. 1	Subtelocentric FIpter=0.14	Medium signal, posterior to 227C. Sex related	0.70 (**±0.10**)	5
420/425C	121F16	*OniX*420 & *OniY*425	7?	Chrom. 2	Subtelocentric FIpter=0.19	Medium signal, anterior to BrainaromS. Sex related	0.66 (**±0.06**)	19
			3	Chrom. 1	Subtelocentric FIpter=0.14	Weak signal, anterior to 227C signal. More likely. Sex related	0.49	1
Brainarom S	115G6	Brain Aromatase	7	Chrom. 2	Subtelocentric FIpter=0.19	Strong signal, posterior to 420/425C on q arm	0.77 (**±0.07**)	10

N/A = Not Available

T = BAC clones identified by Bo-Young Lee and Aimee Howe (Univ. New Hampshire)

C = BAC clones identified by Chuta Boonphakdee (Univ. Stirling)

S = BAC clones identified by Simon Harvey (Univ. Stirling)

Appendix VIII. FISH data from O. karongae, O. mossambicus, O. aureus using probes prepared by NICK TRANSLATION techniques

O. karongae FISH (Nick Translation)

BAC Clone no.	Well ID	Marker/Gene	LG	Physical Map	Chrom. Type (Mitosis) & centromere FIpter	Comments	Mean FIpter (±SD)	Total Measures
12T	D02	Wt1a	7	Chrom. 5	Submetacentric FIpter=0.24	Strong signal, telomeres p arm	0.19 (**±0.04**)	7
17T	A03	UNH995 & UNH104	1	Chrom. 2	Subtelocentric FIpter=0.11	Strong signal, between middle and telomeres q arm	0.64 (**±0.04**)	17
227C	72B20	*OniY*227	3	Chrom. 1	Subtelocentric FIpter=0.14	Strong signal, between middle and telomeres q arm	0.61 (**±0.03**)	10
420/425C	121F16	*OniX*420 & *OniY*425	7?	Chrom. 5	Submetacentric FIpter=0.24	Strong signal, between middle and telomeres q arm	0.61	1
Brain aromS	115G6	Aromatase (brain)	7	Chrom. 5	Submetacentric FIpter=0.24	Strong signal, telomeres q arm	0.76 (**±0.04**)	9

O. mossambicus FISH (Nick Translation)

BAC Clone no.	Well ID	Marker/Gene	LG	Physical Map	Chrom. Type (Mitosis) & centromere FIpter	Comments	Mean FIpter (±SD)	Total Measures
17T	A03	UNH995 & UNH104	1	Small Chrom.	Submetacentric FIpter=0.32	Strong signal, between middle and telomeres of the q arm	0.72 (**±0.03**)	6
227C	72B20	*OniY*227	3	Chrom. 1	Subtelocentric FIpter=0.20	Strong signal, middle of the q arm	0.60 (**±0.03**)	8

O. aureus FISH (Nick Translation)

BAC Clone no.	Well ID	Marker/Gene	LG	Physical Map	Chrom. Type (Mitosis) & centromere FIpter	Comments	Mean FIpter (±SD)	Total Measures
17T	A03	UNH995 & UNH104	1	Small Chrom.	Submetacentric FIpter=0.32	Strong signal, between middle and telomeres of the q arm	0.70 (**±0.11**)	3
227C	72B20	*OniY*227	3	Chrom. 1	Subtelocentric FIpter=0.12	Strong signal, middle of the q arm	0.60 (**±0.04**)	6

T = BAC clones identified by Bo-Young Lee and Aimee Howe (Univ. New Hampshire)

C = BAC clones identified by Chuta Boonphakdee (Univ. Stirling)

S = BAC clones identified by Simon Harvey (Univ. Stirling)

Appendix IX. FISH data from O. mortimeri, T. rendalli, T. zillii, S. galilaeus using probes prepared by NICK TRANSLATION techniques

O. mortimeri FISH (Nick Translation)

BAC Clone no.	Well ID	Marker/Gene	LG	Physical Map	Chrom. Type (Mitosis) & centromere FIpter	Comments	Mean FIpter (±SD)	Total Measures
17T	A03	UNH995 & UNH104	1	Small Chrom.	Submetacentric FIpter=0.32	Strong signal, between middle and telomeres of the q arm	0.69 (**±0.07**)	6
227C	72B20	*OniY*227	3	Chrom. 1	Subtelocentric FIpter=0.12	Strong signal, middle of the q arm	0.59 (**±0.03**)	8

T. rendalli FISH (Nick Translation)

BAC Clone no.	Well ID	Marker/Gene	LG	Physical Map	Chrom. Type (Mitosis) & centromere FIpter	Comments	Mean FIpter (±SD)	Total Measures
16T	H02	Insulin	10	Small Chrom.	Submetacentric FIpter=0.33	Strong signal, just after centromere	0.47 (**±0.14**)	8
17T	A03	UNH995 & UNH104	1	Small Chrom.	Subtelocentric/ Submetacentric FIpter=0.30	Strong signal, between middle and telomeres of the q arm	0.71 (**±0.02**)	2
227C	72B20	*OniY*227	3	Chrom. 1	Subtelocentric FIpter=0.25	Strong signal, middle of the q arm	0.61 (**±0.09**)	10

T. zillii FISH (Nick Translation)

BAC Clone no.	Well ID	Marker/Gene	LG	Physical Map	Chrom. Type (Mitosis) & centromere FIpter	Comments	Mean FIpter (±SD)	Total Measures
17T	A03	UNH995 & UNH104	1	Small Chrom.	Submetacentric FIpter=0.33	Strong signal, just after centromere	0.70 (**±0.11**)	6
227C	72B20	*OniY*227	3	Chrom. 1	Subtelocentric FIpter=0.20	One q arm longer than the other on Chromosome 1	0.62 (**±0.06**)	5

S. galilaeus FISH (Nick Translation)

BAC Clone no.	Well ID	Marker/Gene	LG	Physical Map	Chrom. Type (Mitosis) & centromere FIpter	Comments	FIpter Mean	Total Measures
17T	A03	UNH995 & UNH104	1	?		Bad quality and few chromosome spreads available		
227C	72B20	*OniY*227	3	Chrom. 1	Subtelocentric FIpter=0.25	Strong signal, middle of the q arm	0.62 (**±0.04**)	3

T = BAC clones identified by Bo-Young Lee and Aimee Howe (Univ. New Hampshire)

C = BAC clones identified by Chuta Boonphakdee (Univ. Stirling)

Appendix X. O. niloticus measurements from SCs spreads

***O. niloticus* measurements from a SC spread**							
Chrom no.	**SC Total length (µm)**	**Centromere**		**Secondary marks (µm)**	**Range brighter DAPI band (µm)**	**gap (µm)**	**Chrom type**
		µm	**FIpter**				
1	15.28	1.62	0.11	5.36, 7.72, 9.67, 10.73			A/ST
2	9.83	1.95	0.20	3.90, 6.42, 7.80		5.44	ST
3	7.56	1.38	0.18	2.92, 6.09	6.50-7.23		ST
4	7.15	1.38	0.19		5.36-6.34		ST
5	6.82	2.11	0.31	3.00		1.30	SM
6	6.66	0.81	0.12	3.57, 5.12			ST
7	6.58	1.95	0.30	5.28			SM
8	6.58	1.30	0.20		4.79-5.77		ST
9	6.42	1.13	0.18				ST
10	6.42	1.13	0.18	4.47		1.46	ST
11	6.34	2.19	0.35	0.97, 3.00, 4.63		1.46	SM
12	6.34	0.65	0.10	2.03			A/ST
13	6.34	0.97	0.15				ST
14	6.26	0.89	0.14	5.36			ST
15	6.18	0.89	0.14	5.61			ST
16	5.61	0.73	0.13	4.63			ST
17	5.77	0.81	0.14	3.98			ST
18	5.69	1.38	0.24	4.79			ST
19	5.69	0.97	0.17	3.00			ST
20	5.61	1.13	0.20	2.84			ST
21	5.04	0.48	0.10	3.25, 4.63		1.54	A/ST
22	4.47	0.73	0.16		2.60-4.30		ST

A = Acrocentric, ST = Subtelocentric, SM = Submetacentric

Appendix XI. O. karongae measurements from SCs spreads

***O. karongae* measurements from a SC spread**							
Chrom no.	**SC Total length (µm)**	**Centromere**		**Secondary marks (µm)**	**Range brighter DAPI band (µm)**	**gap (µm)**	**Chrom type**
		µm	**FIpter**				
1	14.48	2.04	0.14	6.08			ST
2	10.83	1.60	0.15	4.37			ST
3	9.84	1.49	0.15	2.93			ST
4	9.25	3.37	0.36	4.48			SM
5	8.79	1.82	0.21	5.69		5.03	ST
6	6.46	2.54	0.39	1.60			SM
7	6.46	0.99	0.15	2.38			ST
8	6.35	0.94	0.15	4.70			ST
9	6.29	1.10	0.17	5.63	1.82-2.70		ST
10	6.24	1.00	0.16				ST
11	6.08	2.38	0.39				SM
12	5.97	1.05	0.18				ST
13	5.91	1.38	0.23	4.70			ST
14	5.91	0.72	0.12	3.76			A/ST
15	5.80	1.11	0.19	2.82			ST
16	5.53	1.27	0.23	4.26			ST
17	5.41	1.05	0.19	2.04			ST
18	4.86	0.83	0.17	2.60			ST
19	4.53	1.16	0.26	2.60			ST

A = Acrocentric, ST = Subtelocentric, SM = Submetacentric

LIST OF COMMUNICATIONS

ORAL PRESENTATION:

- **Jose C Mota-Velasco, Tariq Ezaz, Brendan McAndrew and David J Penman** (2005). Genetic sex determination in Nile Tilapia: comparison of a sex-linked marker and progeny testing. In: WAS (Eds) Proceedings of The World Aquaculture 2005, the annual international meeting, May 9-13, 2005, Bali International Convention Center, Nusa Dua, Bali, Indonesia.

POSTER PRESENTATION:

- **Jose C Mota-Velasco, Chuta Boonphakdee, John B Taggart, Brendan J McAndrew and David J Penman** (2006). The development of microsatellites from BAC clones located to chromosome pair 1 in Nile Tilapia. In: IAGA (Eds) Proceedings of the IX International Symposium Genetics in Aquaculture, June 26-30, 2006, Le Corum, Montpellier, France.

PUBLICATIONS:

- **Jose C Mota-Velasco, Konrad Ocalewicz, Rafael Campos-Ramos and David J Penman** (in preparation). Karyotype evolution in Tilapia: analysis of chromosome fusions in *Oreochromis karongae.*

- **Konrad Ocalewicz, Jose C Mota-Velasco, Rafael Campos-Ramos and David J Penman** (in preparation). FISH and DAPI staining of the synaptonemal complex of the Nile tilapia (*Oreochromis niloticus*) allow orientation of the unpaired region of bivalent 1 observed during early pachytene.